Monitoring of response to therapy with Tyrosine kinase inhibitor in Chronic Myeloid Leukemia: Comparative Study of International Scale Normalized Copy Number values of *BCR::ABL1* fusion gene utilizing quantitative Real-time PCR, with other hematological parameters

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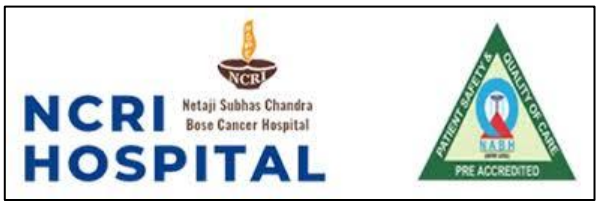
Submitted By

Laiba Usmani
Registration ID: 230020129058
Enrollment ID: 2322210002012

Advisor
Dr. Indranil Dey
Consultant Molecular Pathologist
Netaji Subhas Chandra Bose Cancer Hospital



School of Health Science and Translational Research
Sister Nivedita University
Kolkata

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NCRI HOSPITAL
Netaji Subhas Chandra Bose Cancer Hospital

NETAJI SUBHAS CHANDRA BOSE CANCER HOSPITAL
(A Unit of Himadri Memorial Cancer Welfare Trust)
3081 Nayabad, New Garia, Kolkata, West Bengal 700094
Help Desk - 033 71223000 //Mob: 9874210003 // 9874210007
Website : www.nscri.in • Email : info@nscri.in

Dated: 16/05/2025

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This is to certify that **Ms. LAIBA USMANI** has completed her Project work from **6th January 2025 to 6th April 2025** and presented her thesis on **"Monitoring of response to therapy with Tyrosine kinase inhibitor in Chronic Myeloid Leukemia: Comparative Study of International Scale Normalized Copy Number values of *BCR::ABL1* fusion gene utilizing quantitative Real-time PCR, with other hematological parameters."** for the partial fulfillment of the requirements for the award of the Masters of Science in Biotechnology at Sister Nivedita University, Kolkata.

Dr. Indranil Dey
MBBS, MD (Pathology)
PDF (Molecular Pathology)
Consultant Molecular Pathologist
MCI - 12-45086

Dr. Indranil Dey
Consultant Molecular Pathologist
Molecular Biology Department
Netaji Subhas Chandra Bose Cancer Research Institute
(Unit of Himadri Memorial Cancer Welfare Trust)
Kolkata- 700094

Date- 16/05/2025

Place: Kolkata

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DG Block - 1/2, Action Area I, New Town, Kolkata - 700156, West Bengal, India.

DEPARTMENT OF BIOTECHNOLOGY & DEPARTMENT OF MICROBIOLOGY
জৈব প্রযুক্তি বিভাগ ও অণুজীব বিদ্যা বিভাগ

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Date: 03.06.2025.

Place: SISTER NIVEDITA UNIVERSITY

Signature of Head of the Department

Seal

Head
Department of Biotechnology
Sister Nivedita University
Block DG-1/2, Action Area 1
New Town, Kolkata-700 156

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I hereby declare that the work which is being presented in this dissertation entitled "**Monitoring of response to therapy with Tyrosine kinase inhibitor in Chronic Myeloid Leukemia: Comparative Study of International Scale Normalized Copy Number values of *BCR::ABL1* fusion gene utilizing quantitative Real-time PCR, with other hematological parameters**" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from **06.01.2025 to 06.04.2025** under the supervision of **Dr. Indranil Dey**, Department of Molecular Pathology, Netaji Subhas Chandra Bose Cancer Hospital, Kolkata.

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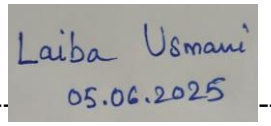
----------  ----------

(Signature of the students with Date)

Name of the Student: Laiba Usmani

Registration no.: 230020129058

Department: Biotechnology

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ABBREVATIONS

Abbreviation	Full Form
CML	Chronic Myeloid Leukemia
Ph	Philadelphia Chromosome
t(9;22)(q34;q11)	Chromosomal Translocation between Chromosome 9 and 22
BCR::ABL1	Breakpoint Cluster Region - Abelson Murine Leukemia Viral Oncogene 1
TKIs	Tyrosine Kinase Inhibitors
MRD	Minimal/ Measurable Residual Disease
qPCR	Quantitative Polymerase Chain Reaction
dPCR	Digital Polymerase Chain Reaction
NGS	Next-Generation Sequencing
FISH	Fluorescence In Situ Hybridization
AI	Artificial Intelligence
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
Ct	Cycle Threshold
ABL1	Abelson Murine Leukemia Viral Oncogene Homolog 1
NCN	Normalized Copy Number
IS	International Scale
CF	Correction Factor
NIBSC	National Institute for Biological Standards and Control
DMR	Deep Molecular Response
MMR	Major Molecular Response
CMR	Complete Molecular Response
EMR	Early Molecular Response
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
CHR	Complete Hematologic Response
TFR	Treatment-Free Remission
ELN	European LeukemiaNet

CBC	Complete Blood Count
BUN	Blood Urea Nitrogen
EDTA	Ethylenediaminetetraacetic Acid
TR	Treatment Responder
TNR	Treatment Non-Responder
UT	Untreated
µL	Microliter
ng/µL	Nanogram per Microliter
QC	Quality Control
CN	Copy Number
G:E	Granulocyte to Erythroid Ratio
SKY	Spectral Karyotyping
TKD	Tyrosine Kinase Domain
FAM	Fluorescein Amidite
SYBR	SYBR Green Dye
MR 3	Molecular Response Level 3
MR 4	Molecular Response Level 4
MR 4.5	Molecular Response Level 4.5
MR 5	Molecular Response Level 5
CBA	Chromosome Banding Analysis
CCyR	Complete Cytogenetic Response
RT-Q-PCR	Real-Time Quantitative Polymerase Chain Reaction
ANOVA	Analysis of Variance

ABSTRACT

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm associated with the Philadelphia chromosome, which is a result of the t (9;22) (q34; q11) translocation and the *BCR::ABL1* fusion gene. The *BCR::ABL1* fusion gene encodes a constitutively active tyrosine kinase that leads to unregulated cell proliferation. Tyrosine Kinase Inhibitors (TKIs) such as imatinib have changed CML from a fatal heath issue to a chronic, manageable disease. However, the importance of minimal residual disease (MRD) remains important to demonstrate effectiveness of treatment, modify therapies, and evaluate treatment-free remission (TFR). In this study, quantitative real-time PCR (qRT-PCR) was performed to identify *BCR::ABL1* transcript levels in peripheral blood samples from patients with CML treated with TKIs. Molecular response was evaluated by calculating the normalized copy number (NCN) and values on the international scale (IS). Molecular data were compared to routine hematological tests. The qRT-PCR assay was sensitive and could detect deep molecular responses such as MR4, MR4.5, and MR5 in some patients. ANOVA demonstrated statistically significant differences were demonstrated for molecular response, based on the different hematological baselines of each patient. This study confirms the superiority of molecular methods compared to conventional methods, using conventional response measures to monitor response to treatment and the disease. There have been improvements, but barriers remain in India, including the cost, access, and standardization of MRD testing. As MRD assessment is critical for personalized CML management and improving long-term outcomes, it is of utmost importance to incorporate MRD evaluation within the standard treatment pathway. Future opportunities may include using digital PCR and what machine learning algorithms might do to evaluate MRD quantification and predictive power.

Keywords:

Chronic Myeloid Leukemia, *BCR::ABL1*, Tyrosine Kinase Inhibitor, Minimal Residual Disease, Real-time PCR, Molecular Monitoring

INTRODUCTION

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder in which the Philadelphia (Ph) chromosome is generated through translocation, t(9;22)(q34;q11) leading to a *BCR::ABL1* fusion gene. This alteration leads to constitutive tyrosine kinase signaling among myeloid cells that cannot control their proliferation. The advent of tyrosine kinase inhibitors (TKIs) has significantly improved the outcome for these patients, leading to CML transforming from a terminal disease to a manageable chronic disease with improved survival[1]. Even so, despite deep molecular responses, residual leukemia may remain, and accurate assessment of Minimal/measurable Residual Disease (MRD) can be difficult without sensitive monitoring techniques.

MRD refers to the small quantity of cancer cells that remain in the body post treatment and are undetectable by standard cytomorphological and cytogenetic techniques. The identification and quantification of MRD has significant implications clinically, as even small quantities of residual disease may lead to relapse. MRD assessment is important to understanding the depth of response, tailoring treatment, and when deciding whether to continue or discontinue therapy altogether[2]. In CML, monitoring MRD is especially salient to evaluating the effect of TKIs, understanding the risk of disease progression, and identifying patients who are candidates for treatment discontinuation based on strict molecular response criteria [1]. MRD monitoring on an ongoing basis has also highlighted the importance of delayed relapse detection, which offers an opportunity for timely therapy and overall better outcomes.

Because of its sensitivity and specificity, quantitative real time polymerase chain reaction (qPCR) for *BCR::ABL1* transcripts is the gold standard for MRD detection. It is capable of picking up one leukemic cell from 10,000 to 100,000 normal cells[3]. Newer technologies, including digital PCR (dPCR) and next-generation sequencing (NGS), are improving the precision of next-generation MRD detection, with even fewer disease burden qualitative changes and an improved reproducibility of reporting. Flow cytometry and other molecular diagnostics also provide complimentary monitoring data for disease monitoring, providing mechanisms for MRD detection. Artificial intelligence and machine learning are increasingly explored for MRD reporting as well to improve data interpretation and predictive accuracy[4].

Even though MRD assessment is advancing globally, there are still challenges concerning standardization and broader implementation of these techniques. In India, MRD monitoring techniques continue to be an area of focus in both clinical and research applications. The routine use of MRD assessment in the management of CML can be impacted in factors such as cost, access to molecular tests, and variability in testing protocols[3]. Additionally, the lack of consistency in MRD guidelines across laboratories can contribute to variability in patient management. Addressing these challenges through standardized protocols, improved uptake of molecular testing, and incorporating MRD into routine clinical practice will assist with developing personalized treatment modifications and ultimately lead to improved outcomes. Here we review the latest advances in MRD detection methods in CML, with a significant focus on their clinical relevance in the context of new technology and implementation challenges in Indian healthcare settings [5]. By discussing the existing and evolving MRD monitoring methods, we aim to provide an overview of MRD monitoring in

relation to CML management, its implications for patient care, and its future potential. We will also discuss the importance of MRD-guided treatment decisions in relation to treatment-free remission (TFR) and long-term disease control.

MATERIALS AND METHODS

Our study was conducted in the molecular pathology laboratory at Netaji Subhash Chandra Bose Cancer Hospital, Kolkata, West Bengal. Consecutive CML patients whose diagnosis was confirmed by hematological and molecular assays, were included in our study over a period of three months from January till March 2025. All patients diagnosed as CML on the basis of clinical and hematological criteria and were positive for *BCR::ABL* by either FISH or qPCR and consented for TKI therapy were included in the study.

The demographic information, clinical and physical examination findings were noted from the medical records of CML patients who either presented at diagnosis or during follow up. The previous hematological and molecular results were also noted. All patients were worked up at presentation with complete blood counts (CBC), baseline coagulogram and for routine biochemical parameters: BUN (blood urea nitrogen), blood sugar, serum creatinine, uric acid and electrolytes). Peripheral blood samples were collected in EDTA for *BCR::ABL* FISH and/ or qPCR. Peripheral blood sample which was collected from these patients were simultaneously analyzed in the hematology lab and molecular pathology lab. The sample collected was first analyzed using automated hematology cell counters (5 part, Sysmex). Based on the total WBC count, appropriate amount of sample was processed for molecular testing which involved RNA extraction from WBCs, RNA quantification and quality check and quantitative *BCR::ABL1* analysis utilizing real time PCR technique.

Sample collection:

A total of 8-10cc peripheral blood was collected and equally divided in 3 EDTA vacutainers. One sample was sent for hematological analysis while the remaining samples were processed for molecular testing.

RNA isolation:

Total RNA was extracted from blood sample using the NucleoSpin® RNA Blood Kit. This kit processes by direct total blood lysis from 200 – 400 µL whole blood collected in standard (e.g., EDTA) blood collection tubes. One of the most important aspects in RNA purification is to prevent RNA degradation during the isolation. With the NucleoSpin® RNA Blood method, leukocytes (the main source of RNA in whole blood) and other blood cells, are lysed by incubating the whole blood in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases (which are present in virtually all biological materials) and creates appropriate binding conditions that favor adsorption of RNA to the silica membrane. A complex selective erythrocyte lysis and preparation of a leukocyte pellet is not necessary. Contaminating DNA, which is also bound to the silica membrane, is removed by a recombinant DNase solution which is directly applied onto the silica membrane during the preparation. Simple washing steps with two different buffers remove salts, metabolites, and macromolecular cellular components. Pure RNA is finally eluted under low ionic strength conditions with RNase-free H2O. The RNA preparation using NucleoSpin® RNA Blood kits is performed at room temperature. To ensure RNA stability, RNA is kept frozen at -20°C for short-term or -70°C for long-term storage.

RNA isolation process:

1. **Lyse blood**

- Take 200µL whole blood in a Collection Tube (2mL, with lid; provided). Add 200 µL Lysis Buffer DL to the tube and close the lid. Mix. If necessary, shortly spin to clean the lid.
- Add 5µL Liquid Proteinase K and close the lid. Incubate 3-15 min at room temperature (18-25°C) vigorously shaking the tube on a shaker (e.g., Eppendorf Thermoshaker, 1,400 rpm).
- Centrifuge briefly to clean the lid (~ 1 s at ~ 2,000 x g). Short spin only.

2. **Adjust RNA binding conditions**
    - Add 200µL 70% ethanol to the tube and mix vigorously.
    - Centrifuge briefly to clean the lid (~ 1s at ~ 2,000 x g). Short spin only ~1sec.
3. **Bind RNA**
    - Adjust pipette to 610µL and transfer lysate into a NucleoSpin® RNA Blood Column placed in a Collection Tube.
    - Centrifuge 30 s at 11,000 x g. Discard flow-through and Collection Tube. Place the column in a new Collection Tube (2mL; provided).
4. **Desalt silica membrane**
    - Add 350µL MDB (Membrane Desalting Buffer) onto the column and centrifuge 30s at 11000 x g.
5. **Digest DNA**
    - Add 95µL rDNase onto the column. Incubate at room temperature for 15 min.
6. **Wash and dry silica membrane**

    <u>1st wash</u>
    - Add 200µL Buffer RB2 to the NucleoSpin® RNA Blood Column. Centrifuge for 30s at 11,000 x g. Discard flowthrough and Collection Tube and place the column into a new Collection Tube (2mL; provided)

    <u>2nd wash</u>
    - Add 600µL Buffer RB3 to the NucleoSpin® RNA Blood Column. Centrifuge for 30s at 11,000 x g. Discard flowthrough and place the column into a new Collection Tube (2mL; provided).

    <u>3rd wash</u>
    - Add 250µL Buffer RB3 to the NucleoSpin® RNA Blood Column. Centrifuge for 2min at 11,000 x g. In this step, ethanol is removed from the column. Place the column into a nuclease-free Collection Tube (1.5mL, supplied) and discard the Collection tube with flowthrough from the previous step.

    [If for any reason the liquid level in the Collection Tube has reached the NucleoSpin® RNA Blood Column after centrifugation, discard flow-through, and centrifuge again.]
7. **Elute RNA**
    - Add 60µL RNase-free H2O (supplied) onto the column and centrifuge 30s at 11,000 x g. The RNA is eluted into the Collection Tube.

Figure 1: NucleoSpin Blood RNA Extraction Kit

- **Evaluation of Extracted RNA**
  Techniques used for quality and quantity check of RNA are as follows:

1. **Gel electrophoresis (1% Agarose gel):** Two bands **(28S:18S)** observed in the ratio of **2:1** is a good indication of intact RNA. (**Figure 2**)

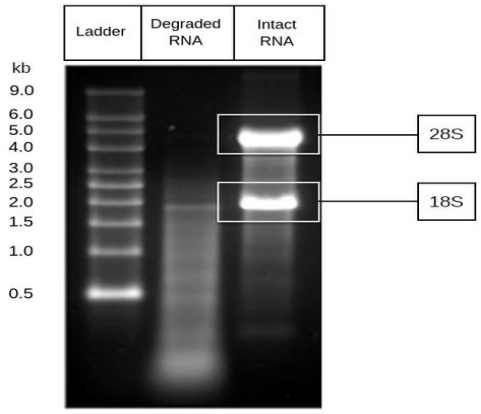


Figure 2: Gel electrophoresis of RNA

2. **Spectrophotometer reading**:

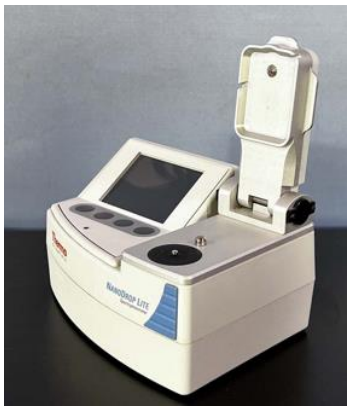


Figure 3: NanoDrop lite

Interpretation:

i. ng/uL: Sample concentration in ng/uL based on absorbance at 260 nm and the selected analysis constant.

ii. 260/280: Ratio of sample absorbance at 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of RNA. A ratio of **~2.0** is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm.

iii. 260/230: Ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of **1.8-2.2**. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

Real Time PCR Process:

All RNA samples which passed the quality and quantity QC were processed for Quantitative *BCR::ABL1* quantification by real time PCR using Biorad CFX96 Real time PCR and TRUPCR ® *BCR ABL1* Kit (3B Blackbio, India). This kit intended for the quantitative detection and differentiation of BCR-ABL1 fusion gene transcript Major (M), minor (m), and micro (μ) in bone marrow or peripheral blood samples.

Figure 4: Bio-Rad qPCR Thermal Cycler

TRUPCR® *BCR-ABL1* Kit is an RT-qPCR assay based on oligonucleotide hydrolysis principle which allows higher specificity and sensitivity. In real-time PCR, the fluorescent signal is generated from the presence of an oligonucleotide probe specific for target DNA sequence. The probe contains a fluorescent dye molecule on its 5' end and a quencher molecule on its 3' end. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Fluorescence resonance energy transfer (FRET). The probe hybridizes with one of the chains of the amplified fragment. During synthesis of a complementary chain, Taq DNA polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. As a result, the fluorescent dye and quencher dye are separated, and the total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The fluorescent signal is measured in each cycle of reaction, and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number of DNA copies in a sample and its value allows qualitative comparisons of analyzed and control samples.

TRUPCR® *BCR::ABL1* detection is a Real-Time amplification test for the quantitative detection of *BCR-ABL1 b2a2, b3a2, e1a2* and *e19a2* fusion transcripts in bone marrow or peripheral blood samples. It has two-step protocol in which total RNA is reverse-transcribed, and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe of *BCR-ABL1* (Major, Minor and Micro) and ABL1. The probe binds to the amplicon during each annealing step of the PCR. When the Taq extends from the primer bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the Taq DNA polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them and leading to an increase in fluorescence from the reporter and a decrease in fluorescence from the quencher.

c-DNA synthesis process:

cDNA is synthesized from extracted RNA by reverse transcriptase PCR (RT-PCR) technique.

Reverse Transcription PCR Protocol

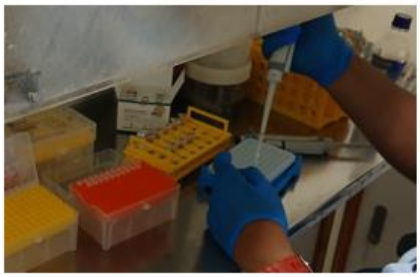
1. **Table 1: REACTION PREPARATION:**

Name of the Reagent	Quantity per reaction
RRT 1	8 µl
RRT 2	1.5 µl
RRT 3	5 µl
RRT 4	0.5 µl
Sample RNA*	1 µg
Total reaction volume	**25 µl**

2. **PROGRAM SET UP:**
   Table 2: Following setting for Temperature Profile of the thermal cycler is designated.

Step	Temperature, °C	Time	Cycles
1	25	10 min	1
2	47	60 min	1
3	70	05 min	1

(A)



(B)

Figure 5: (A) Preparation od reaction components for reverse transcription polymerase chain reaction (RT-PCR) under aseptic conditions. (B) Reverse transcriptase PCR thermal cycler used for amplification and quantitative analysis of target nucleic acid sequences

Real Time PCR Protocol

1. **REACTION PREPARATION FOR SAMPLES:**
   The TRUPCR® *BCR-ABL1* kit is a multi-tube format kit. Hence, each sample would be split in separate tubes for separate transcript detection. For all three transcripts four separate tubes had to be run for Major, Minor, Micro *BCR-ABL1* and *ABL1*.

Table 3: We prepared the PCR Mix as follows: following 4 columns represents four tubes for each sample:

Name of the Reagent	MBCR-ABL1 (Major)	mBCR-ABL1 (Minor)	µBCR-ABL1 (Micro)	ABL1
Multiplex Master Mix	10 µl	10 µl	10 µl	10 µl
Major BCR-ABL1 Primer probe mix	1 µl	-	-	-
Minor BCR-ABL1 Primer probe mix	-	1 µl	-	-
Micro BCR-ABL1 Primer probe mix	-	-	1 µl	-
ABL1 Primer probe mix	-	-	-	1 µl
Sample cDNA	5 µl	5 µl	5 µl	5 µl
Nuclease free Water	4 µl	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**

2. **REACTION PREPARATION FOR STANDARDS:**

Table 4: We prepared six reactions for *BCR-ABL1* Standards.

Name of the Reagent	STD 1 (10^6 copies)	STD 2 (10^5 copies)	STD 3 (10^4 copies)	STD 4 (10^3 copies)	STD 5 (10^2 copies)	STD 6 (10^1 copies)
Multiplex Master Mix	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
Major BCR-ABL1 Primer probe mix	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Standards	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
Nuclease free Water	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**	**20 µl**

Table 5: Then we prepare three reactions for *ABL1* standards.

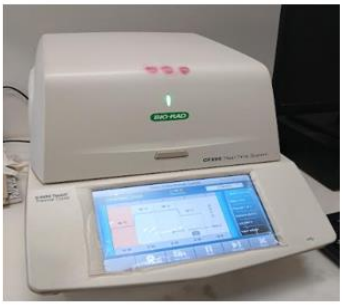
Name of the Reagent	STD 1 (10^6 copies)	STD 2 (10^5 copies)	STD 3 (10^4 copies)
Multiplex Master Mix	10 µl	10 µl	10 µl
ABL1 Primer probe mix	1 µl	1 µl	1 µl
Standards	5 µl	5 µl	5 µl
Nuclease free Water	4 µl	4 µl	4 µl
Total reaction volume	**20 µl**	**20 µl**	**20 µl**

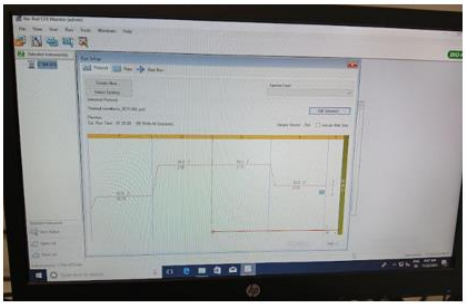
3. **PROGRAM SET UP:**

Table 6: We define the following setting for Temperature Profile and Dye Acquisition.

Step	Temperature, °C	Time	Dye Acquisition	Cycles
1	94	10 min	-	1
2	94	15 sec	-	45
	60	01 min	Yes	

(A)





(B)

Figure 6: (A) Real-time PCR (qPCR) thermal cycler utilized for amplification and fluorescence-based detection of target genes. (B) Amplification plot interface displaying real-time fluorescence signals during qPCR run.

4. CHANNEL SELECTION

Table 7: Then we define the following setting for channel selection.

Detection	Detector channel	Reporter	Quencher	Gain Setup
BCR - ABL1	Green	FAM	None	Auto
ABL1	Green	FAM	None	Auto

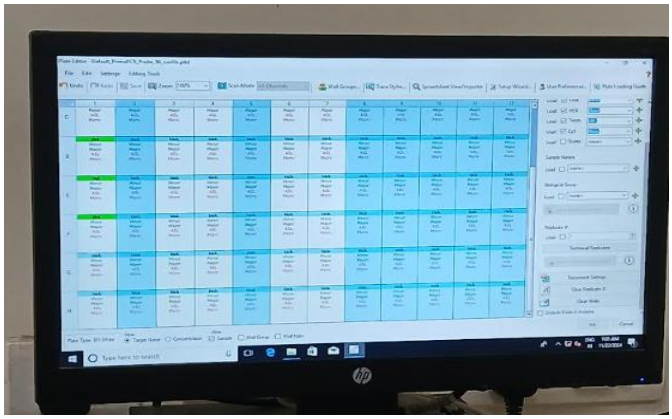


Figure 7: Software interface showing sample setup and configuration for multiplex direction of *BCR-ABL1* and *ABL1* transcripts using FAM-labeled probes.

Quantitative Result Analysis

The Quantitative result analysis was done utilizing the standard curve method and Standard Curve was generated for both *ABL1* and *BCR::ABL1*. Standard Curve was derived from 6 dilution points for *BCR::ABL1* and 3 dilution points for *ABL1* (a minimum of three dilution points for each) as per standard recommendations. As standards were of tenfold dilution, the theoretical slope of the curve was calculated to be –3.3; however, a slope between –3.0 and –3.9 was considered acceptable as long as R^2 was >0.95. We however, maintained a R^2 value of >0.98 to generate precise results.

Analysis of the results were performed under the guidance of trained personnel and the test results were evaluated by molecular pathologist, taking the patient's clinical findings and the results of other tests into consideration. The values for unknown samples were depicted in the result column in copies/µL in FAM Channel for *BCR::ABL1* and *ABL1*. For samples showing no amplification in *BCR::ABL1* we checked if amplification was present for *ABL1* (housekeeping gene) (absolute *ABL1* copy number ≥ 10,000 copies). This was done to avoid false negative results due to the quality of RNA, and only if *ABL1* was amplified

satisfactorily, then the results were considered. We also checked that the negative control did not show any value in the result column for both *BCR::ABL1* and *ABL1*. Any amplification in the negative control would indicate cross contamination. Utilizing the standard curves we are able to determine the copy number (CN) of the *BCR::ABL1* fusion (major, minor/ micro) transcript and the transcripts of the housekeeping genes *ABL1*. The *ABL1* copy numbers (ABL1 CN) and *BCR::ABL1* absolute copy numbers (*BCR::ABL1* CN) obtained in the test results were then used to calculate the Normalized copy number (NCN) for samples. The ratio of these CN values gives the normalized copy number (NCN):

$$\text{NCN (\%)} = \frac{\textit{BCR::ABL1}\ \text{NCN} \times 100}{\textit{ABL1}\ \text{NCN}}$$

Subsequently a lot specific Correction Factor (CF) was provided by the kit manufacturers which was determined using secondary standards calibrated against First WHO International Genetic Reference Panel for quantitation of *BCR ABL1* translocation by RQ-PCR (NIBSC code: 09/138). To convert result in international scale (IS) Normalized copy number (NCN) % following formula was used.

IS NCN (%) = NCN % x Lot specific CF (CF for our kit was **0.733**)

Measuring Molecular Response

Table 8: After calculating the IS NCN % the patients DMR (Deep molecular response) status was ascertained as per the chart below.

Sr No.	*BCR::ABL* Copies	*ABL* Copies	DMR status	Remarks
MAJOR MOLECULAR RESPONSE (MMR)				
1	> 0.1 %	< 10000	Inconclusive	Poor Quality Sample
2	> 0.1 %	> 10000	No MMR	Major Molecular Response not achieved
3	≤ 0.1 %	< 10000	Inconclusive	Poor Quality Sample
4	≤ 0.1 %	> 10000	MMR (MR 3)	Major Molecular Response achieved with ≥3-log reduction from IRIS baseline
DEEP MOLECULAR RESPONSE (DMR) IN CASE OF DETECTABLE DISEASE				
1	≤ 0.01 %	< 10000	Inconclusive	Poor Quality Sample
2	> 0.01 %	> 10000	No DMR	Deep Molecular Response not achieved
3	≤ 0.01 %	10000-31999	DMR-4	Deep Molecular Response achieved with ≥4-log reduction from IRIS baseline
4	≤ 0.0032 %	32000-99999	DMR-4.5	Deep Molecular Response achieved with ≥4.5-log reduction from IRIS baseline
5	≤ 0.001 %	≥100000	DMR-5	Deep Molecular Response achieved with ≥5-log reduction from IRIS baseline
DEEP MOLECULAR RESPONSE (DMR) IN CASE OF UNDETECTABLE DISEASE				
1	Not Detected	< 10000	Inconclusive	Poor Quality Sample

2	Not Detected	10000–31999	DMR-4	Deep Molecular Response achieved with ≥4-log reduction from IRIS baseline.
3	Not Detected	32000–99999	DMR-4.5	Deep Molecular Response achieved with ≥4.5-log reduction from IRIS baseline
4	Not Detected	≥100000	DMR-5	Deep Molecular Response achieved with ≥5-log reduction from IRIS baseline

After ascertaining the DMR status the response to therapy with TKI were ascertained as defined by ELN guidelines. A database was created in MS Excel and the data was used for statistical analysis.

STATISTICAL ANALYSIS

Statistical analysis of experimental data was done to see whether the differences among groups were significant. One-way analysis of variance (ANOVA) was done manually for each dataset, providing a means of assessing whether the means of each group differed significantly from one another. For the manual analysis, we calculated the total sum of squares (SST), sum of squares between groups (SSB), and sum of squares within groups (SSW), and calculated the mean squares (MSB and MSW). The F-values were scored as the mean square between groups over the mean square within groups (F=MSB/MSW). They were then converted to an estimated p-value using the online stat calculator from GraphPad (https://www.graphpad.com/quickcalcs/pvalue1/), which took the F-ratios and converted them to p-values based on the degrees of freedom established by each dataset, ensuring accurate statistical representation. The F-ratio collected in the ANOVA analysis was considered statistically significant if the p-value was less than 0.05 with less than 5% probability that the differences between groups occurred by chance. This approach bolstered the reliability of the statistical conclusions drawn from our studies.

REVIEW OF LITERATURE

1.Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a form of cancer that originates in the bone marrow and whose essential event is the proliferation of myeloid cells. CML affects mostly, but not exclusively adults. It can arise at any age. John Hughes Bennett, a Scottish physician, made a description of the disease in 1845 that included excess white blood cells but at that time leukemia was not differentiated from other diseases. With the emergence of research and use of the term Chronic Myeloid Leukemia (CML) in the early 1900s, scientists and clinicians were distinguishing CML from acute leukemias, observing that CML was a slower developing disease that primarily afflicted adults, while aggressive acute leukemia was more prevalent in children.

The essential genetic defect (related to CML) is an alteration recognized as the Philadelphia chromosome, which is the functional fusion of the *BCR* gene on chromosome 22 and *ABL1* gene on chromosome 9.[5]. The presence of this gene rearrangement leads to the production of a protein that is responsible for the uncontrolled proliferation of white blood cell and encompasses the ongoing production of the cancerous blood cells that disrupt normal blood functions. CML typically has three discernible phases: chronic, accelerated, and blast crisis. When it is not treated, the symptoms of CML worsen over time as the cancer proliferates.

In 1998, the first tyrosine kinase inhibitor (TKI), Imatinib (Gleevec), which specifically targeted the *BCR::ABL* protein and inhibits its activity, was developed. Imatinib was a game changer for CML, changing it from a fatal disease to a chronic condition that could be managed for a number of patients [6].

Now most patients with CML are living as if they have a manageable chronic disease. Many patients are expected to live normal lifespans with routine monitoring and treatment. The clinical goal for monitoring CML is to have patients in molecular remission, where the *BCR::ABL* gene is no longer detectable in the blood. Molecular monitoring refers to the monitoring that intercepts the amount of *BCR::ABL* fusion gene and activity. The two most common modalities for monitoring therapy are quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR).

1. a. CML in World & India:

CML accounts for approximately 15% of all leukemia cases worldwide, and the annual incidence is approximately 1–2 per 100,000 people. It is most often diagnosed in adults, especially those aged between 45 and 55. The age at diagnosis is usually around 50 to 60 years. Childhood occurrences are quite rare, accounting for around 2-3% of all CML cases. It is more common in men than in women. The disease comes into being in three stages: chronic phase, with asymptomatic symptoms, accelerated phase, characterized by the deterioration of blood counts, and blast crisis, which is the acute and aggressive phase (akin to acute leukemia). In the absence of treatment, CML can movements rapidly and become life-threatening [7]. The pathophysiologic basis of CML is genetic mutations causing unlimited growth of myeloid cells (a white blood cell) and abnormal blood cell formation.

The most characteristic feature of CML is the Philadelphia chromosome, a distinct genetic anomaly in leukemia cells' DNA. It occurs as a result of a translocation between

chromosomes 9 and 22, designated as t(9;22)[8]. The *BCR::ABL* fusion protein creates an aberrant tyrosine kinase. In normal circumstances, tyrosine kinases take part in signaling pathways that control cell growth, survival, and differentiation. *BCR-ABL* protein contains a hyperactive tyrosine kinase activity, which results in the persistent activation of a number of signaling pathways such as MAPK, PI3K/Akt and JAK-STAT pathways. The aberrant signaling produces both autocrine (stimulating self) and paracrine (stimulating nearby cells) effects on cell growth and survival, perpetuating the proliferation cycle [9]. Tyrosine kinase inhibitors (TKIs) revolutionized the treatment algorithm of chronic myeloid leukemia (CML) in the past two decades, with the most well-known being imatinib (Gleevec).

Prior, to TKIs, the median survival time for CML patients was merely 3–5 years, with treatment modalities limited to chemotherapy and bone marrow transplantation. TKIs have transformed CML into a seemingly chronic illness, with a 10-year survival rate of >80-90% in developed countries [10]. First-line TKIs, such as imatinib, dasatinib, and nilotinib, are presently available to block this *BCR::ABL1* fusion protein and manage the disease process. Long-term, the original mutation responsible for the *BCR::ABL* fusion gene may result in additional genetic mutations and clonal evolution of leukemia cells. These secondary mutations may lead to more aggressiveness of the disease and drug resistance (particularly tyrosine kinase inhibitors such as imatinib).

For these patients who are resistant or intolerant to first-line TKIs, new-generation drugs (such as bosutinib, ponatinib) could be used. Advanced disease patients don't gain any advantage from TKIs, therefore allogeneic stem cell transplantation is utilized [11]. In India, CML is a different scenario compared to the Western world, where it accounts for 15% to 20% of all types of leukemia. Among Indians, the rate of CML in leukemia has been estimated as high as 30% and 60%.

The incidence of CML is estimated to be 0.8-2.2 per 100,000 population per year; the actual incidence is likely to be higher due to underreporting and the absence of any national cancer registry [12]. The age of patients at diagnosis with CML in India is much younger than in the Western world, typically in the late 30s and early 40s; thus, the fact that it predominantly occurs in working-age individuals places a huge socio-economic burden upon the population [13]. Cost and access rank as among the largest variables influencing CML management in India. Imatinib was originally highly costly and imposed heavy economic costs on patients until the advent of generics. Despite this, second- and third-generation TKIs like dasatinib and nilotinib are still highly costly and inaccessible to a vast majority of patients.

Potentially curative bone marrow transplantation can be performed only in specialized centers and remains economically beyond the means of most patients in India [13].

1.b. CML Pathogenesis

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm defined by the presence of the Philadelphia chromosome, which originates from a reciprocal t(9;22)(q34;q11) translocation. The t(9;22) produces the *BCR::ABL1* fusion gene encoding a tyrosine kinase that is constitutively active, leading to the proliferation of myeloid cells without control. CML's tyrosine kinase homogeneously activates several downstream signaling pathways to support cell proliferation and suppress apoptosis [14].

The *BCR::ABL1* chimeric protein activates numerous signaling cascades resulting in aberrant signal transduction pathways which ultimately lead to leukemogenesis. Furthermore, *BCR::ABL1* inhibits mechanisms of DNA damage response causing genomic instability and also interferes with the integrin and chemokine signaling thereby facilitating leukemic cell escape from immune detection [15].

CML is also marked by progression from chronic phase (with comparatively normal differentiation) to accelerated and ultimately blast crisis phase (transformation into acute leukemia) which mainly occurs due to acquisition of additional mutations and mounting genetic abnormalities [16].

Philadelphia Chromosome:

The Philadelphia chromosome is a short chromosome 22 due to a reciprocal translocation between chromosomes 9 and 22, defined as t (9; 22) (q34; q11). This is a characteristic genetic abnormality of chronic myeloid leukemia and has been observed in certain instances of acute lymphoblastic leukemia and acute myeloid leukemia [17][18]. The chromosome was identified as early as 1960, when Nowell and Hungerford originally reported a small abnormal chromosome in CML patients [17]. The translocation forms the *BCR::ABL1* fusion gene, the product of which encodes a constitutively active tyrosine kinase with a central role in leukemogenesis because it can induce self-sustaining cell growth, suppress apoptosis, and modify cell adhesion [19].

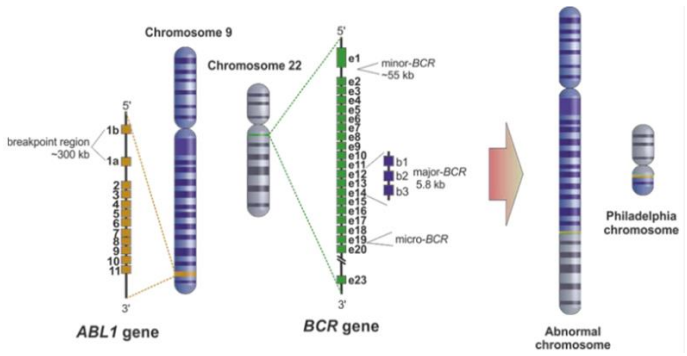


Figure 8: Philadelphia chromosome. The Philadelphia chromosome results from the translocation between the Abelson murine leukemia (*ABL1*) gene on chromosome 9 with the breakpoint cluster region (*BCR)* gene on chromosome 22. Three break point regions might be involved in this translocation: (i) intron 13 or 14 of *BCR*, named major breakpoint (*M-BCR*), (ii) intron 1 of BCR, named minor breakpoint *(m-BCR*), and (iii) exon 19 of *BCR*, named μ breakpoint (μ-BCR). Concerning ABL1, the breakpoint usually involves the region between exons 1b and 2.[20]

The *BCR::ABL1* fusion gene results from juxtaposing the *BCR* gene from chromosome 22 and the *ABL1* gene from chromosome 9[20]. Depending on the precise breakpoint within the *BCR* gene, different isoforms of the *BCR::ABL1* protein can be produced: p190, p210, and p230. The p210 isoform is most commonly associated with CML, whereas p190 is predominantly found in Ph-positive ALL. The p230 variant, although rare, is linked with a more indolent form of the disease. *BCR::ABL1* exhibits persistently high tyrosine kinase activity, leading to the activation of multiple signaling pathways, including JAK-STAT, PI3K-AKT-mTOR, and RAS-MAPK. These pathways promote malignant transformation by enhancing proliferation, survival, and genomic instability [18]. Furthermore, *BCR::ABL1*

alters the bone marrow microenvironment, contributing to leukemia stem cell maintenance and disease progression.

CML goes through three different phases. The chronic phase is characterized by the accumulation of mature granulocytes while symptoms are minimal. The accelerated phase is characterized by increased blasts, worsening cytogenetic abnormalities, and therapy resistance. The blast crisis, resembling acute leukemia, presents a predominance of either myeloid or lymphoid blasts [21]. Additional mutations such as *IKZF1*, *CDKN2A/B* deletions, and *TP53* mutations often support the progression of the disease and resistance to therapy [19].

While Ph is usually associated with CML, it is also found in 11-29% of adult ALL cases, as well as in a smaller fraction of AML cases. In Ph-positive ALL, the p190 isoform of *BCR::ABL1* predominates and leads to a more aggressive course of the disease with a poorer prognosis as compared to Ph-negative ALL. In AML, Ph is rarely detected; it could suggest some unique biological status that would demand specific attention in therapy [17].

The introduction of TKIs, such as imatinib, represents a paradigm shift in the therapy of Ph-positive leukemias. Imatinib binds to the ATP-binding site of *BCR-ABL1*, inhibiting the kinase activity of this oncoprotein and, in turn, upstream kinase signaling [18]. Second- and third-generation TKIs such as dasatinib, nilotinib, bosutinib, and ponatinib have been made to counter the effect of resistance mutations as T315I. However, leukemia stem cells can persist in the presence of TKIs [21]. It is thus deemed of utmost necessity to develop additional treatment strategies such as combination therapies, allogeneic HSCT, and new agents targeting alternative signaling pathways to eradicate them [19].

It is among the most well-defined genetic alterations in hematologic neoplasia. Although TKIs have dramatically enhanced outcomes for Ph-positive leukemia patients, challenges remain in the form of drug resistance and minimal residual disease. Research into targeted therapies and combination approaches is still ongoing, raising hopes for better treatment and, possibly, cures [19].

1. c. Phases of CML:

Chronic myeloid leukemia (CML) is defined by three phases in disease progression: **chronic phase, accelerated phase, and blast crisis phase**. The classification of these phases is based on the proportion of blast cells present in the peripheral blood and bone marrow, as well as the associated clinical symptoms. The progression from one state to another are associated with a gradually worsening condition along with the increased improbability that standard therapies will benefit the patients [22].

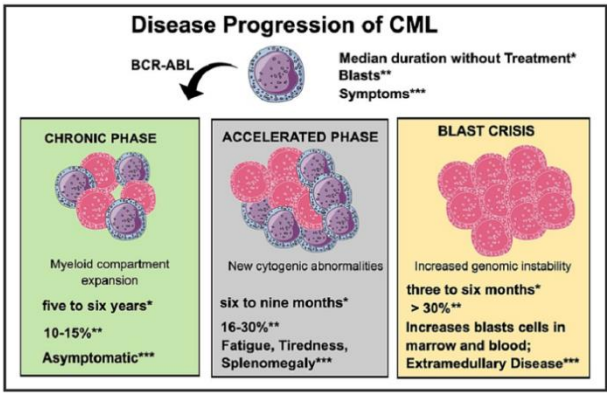


Figure 9: Phases of CML. CML: Chronic myeloid leukemia, BCR-ABL: breakpoint cluster region gene-Abelson proto-oncogene. *Median duration without treatment; **percentage of blasts; ***symptoms.[23]

Chronic Phase: It is the earliest and most prolonged stage of CML, accounting for an estimated 85 to 90% of cases at diagnosis [23]. It is characterized by the slow accumulation of mature and immature myeloid cells in the bone marrow and peripheral blood. In this phase, the percentage of blast cells in the peripheral blood or bone marrow stays below 10%. Clinically, the majority of cases present either with mild and nonspecific symptoms, such as fatigue, weight loss, or night sweats, and splenomegaly, or they're asymptomatic. This phase shows good response to TKIs, such as imatinib, dasatinib, and nilotinib. With good treatment and periodic monitoring, patients in this phase can control the disease indefinitely and enjoy a near normal life expectancy[24].

Accelerated Phase: This phase results from disease progression and is characterized by the increased proliferation of immature myeloid cells, indicating first-line treatment failure. The accelerated phase is defined by an increase in blast cells to between 10% and 19% in the peripheral blood or bone marrow. The accelerated phase is characterized by a 10 to 19% increase in blast cell percentage in the blood or bone marrow. The signs and symptoms are more pronounced; for example, persistent fever, night sweats, unintentional weight loss, worsening anemia, and splenomegaly. Devastatingly, some patients may show an increasing white blood cell count even on treatment. Here, first-generation TKIs are less effective, thus requiring second- or third-generation TKIs like bosutinib and ponatinib. Diagnosis is confirmed when blast cells constitute 20% or more of the cells in the blood or bone marrow [16].

Blast Phase: This is the most advanced and aggressive phase of CML, with a prognosis and outlook comparable to that of acute leukemia. An uncontrolled proliferation of blast cells leads to severe marrow failure and systemic complications in these patients. It is diagnosed when the blast cell percentage in blood or bone marrow is 20% or more[16]. Patients in this phase have severe infections, episodes of recurrent bleeding, extreme fatigue, bone pain, and organ infiltration, owing to the dramatically high blast cell counts. Life-threatening complications are considerably more likely. This phase has poor responses to TKIs, and management requires intensive chemotherapy, targeted therapies, and consideration for allogeneic HSCT. The prognosis is severely dismal, with most patients living only for a few months without aggressive intervention[25].

Based on the classification of CML into three phases, prognosis and treatment decisions can be determined. Survival outcomes following the chronic phase are much better with an early diagnosis and introduction of TKI therapy. Regular molecular monitoring of *BCR-ABL1* transcript levels plays a crucial role in monitoring disease progression and detecting resistance mutations[24]. Upon progressing to the accelerated or blast phases of the disease, these individuals require early interventions to ensure a better therapeutic outcome and to stymie the further deterioration of their condition.

1. d. Clinical Features:

Many patients may be asymptomatic at the time of diagnosis, with CML becoming a chance finding following routine blood tests. These blood tests show generally an increased white blood cell count (leukocytosis), often with a predominance of myeloid cells, and occasionally thrombocytosis, that is, an increased platelet count. However, with disease progression, a spectrum of symptoms starts to develop in patients[26].

Common symptoms of CML include persistent fatigue and weakness, which result from anemia and the increased metabolic demands of the excessive number of abnormal cells. Another common feature is splenomegaly or an enlarged spleen, causing abdominal discomfort or pain; thus, patients may also complain of a sensation of fullness or early satiety due to an enlarged spleen[27]. Night sweats and occasional low-grade fever may occur because of the disease itself or secondary infections. Unintentional weight loss may also occur, stemming from increased energy expenditure and metabolic changes due to the disease. Another symptom experienced by some patients is bone pain attributed to some proliferation of myeloid cells in the marrow[28].

Though there are elevated white blood cell counts, CML patients may have a higher vulnerability to infections as a result of their impaired functionality. Other complications due to thrombocytosis include thrombosis (clots) or bleeding due to dysfunctional platelets. As CML develops, it can also enter the second phase of accelerated CML with abundant immature cells, or blasts in the blood or the bone marrow. The disease finally goes into a blast crisis in which features of acute leukemia manifests, due to increase in blast cells (over 20% in the blood or bone marrow). Symptoms during this phase include intense fatigue, fever, and increased bleeding or bruising[26].

Complete blood counts in CML usually show leukocytosis, with a left shift; with increased numbers of immature myeloid cells. There could be anemia and even thrombocytosis. Bone marrow biopsy in these patients may show hyper cellularity with increased numbers of myeloid cells. Confirmation of diagnosis requires cytogenetic analysis that reveals the Philadelphia chromosome or the *BCR-ABL* fusion gene protein[4].

Long-term complications of CML and its treatment may present with side effects of tyrosine kinase inhibitors such as gastrointestinal disorders, skin rashes, and later possible heart trouble. There is also the emergence of a secondary malignancy due to treatment over long periods[27].

CML is a complex disease with an array of clinical features that vary considerably from one patient to another. Early diagnosis and the use of targeted therapies such as TKIs, including imatinib, have significantly improved the prognosis for patients with CML; many can live long and fulfilling lives. For patients to receive the best care, they will need ongoing monitoring and management for any symptoms and complications that may arise[11].

2. CML lab investigation:

Laboratory investigations are of utmost significance in the diagnosis, staging, and monitoring of CML, such as hematological, biochemical, cytogenetic, and molecular techniques to help ascertain disease burden, response to therapy, and potential progression into advanced phases[5].

2. a. Complete Blood Count:

A complete blood count (CBC) is the initial and one of the most important diagnostic tests in suspected cases of CML. The CBC findings often reveal moderate leukocytosis in excess of 100,000/mm^3. A peripheral blood smear shows a differential count on the left shifted side: that is, an increase in immature myeloid precursors such as myelocytes, metamyelocytes, and promyelocytes within the granulocytic lineage. These features of peripheral blood smear are

often referred to as the "myelocyte bulge." This is associated with a leukocytosis, with white blood cell counts ranging from about 50,000/mm³ to >300,000/mm³. CML is frequently associated with the presence of basophilia and eosinophilia, which allow differentiation from reactive leukocytosis. Usually, there is also normocytic or normochromic anemia because of bone marrow suppression and ineffective erythropoiesis [29].

Thrombocytosis elevates the platelet counts and is observed in some patients with early cases of the disease, while increasingly severe cases result in thrombocytopenia. The range of increase in platelets can vary for different patients, and in some cases, count levels can go above 1,000,000/mm³. Important findings in the CBC of CML patients are absolute neutrophilia, usually with hyper segmented neutrophils [30].

While leukocytosis can be seen in other conditions, the characteristic left shift in the granulocytic lineage along with basophilia, sets it apart from other conditions. In CML, the leukocyte alkaline phosphatase (LAP) score is typically low, whereas it is characteristically elevated in a leukemoid reaction.

Follow-up of CBC parameters is also important in assessing the response to treatment, since with adequate treatment there will be a gradual normalization in all cell populations [29].

Moreover, further information relating to the disease progress can be gathered from a close morphological study of the peripheral blood smear. An increase of circulating blast cells might indicate that the disease has transformed into accelerated or blast phase. Regular monitoring of CBC will not only assist in deciding the therapy to be undertaken on a case-to-case basis but will also help spot early signs of resistance or possible relapse of the disease. Persistent eosinophilia and basophilia in the treated disease could allude to an inadequate response, deserving further molecular and cytogenetic analyses [30].

2. b. Bone Marrow:

A bone marrow aspiration and biopsy can be of significant help in making known the mechanism of the disease and staging CML. The marrow in CML during the chronic phase is usually hypercellular and the myeloid-to-erythroid (M: E) ratio is often greater than 10:1. There is a decrease in the number of erythroid precursors, while megakaryocytes may show abnormal clustering and hyper lobulation. Granulocytic hyperplasia is another prominent

feature with a background of increased myeloid precursors at various stages of differentiation. Typical symptoms of CML include persistent fatigue and weakness, primarily caused by anemia and the elevated metabolic demands of the excessively produced leukemic cells [31].

In certain cases, reticulin staining may reveal increased fibrosis within the bone marrow. Marrow fibrosis has been associated with disease progression and poor prognosis. Bone marrow analysis is a valuable tool in distinguishing between CML and other myeloproliferative neoplasms such as polycythemia vera or primary myelofibrosis. Cytogenetic analysis of bone marrow cells will detect additional chromosomal abnormalities, known as clonal evolution, which may signal disease progression. Certain anomalies have been related to transformation into accelerated or blast phase such as trisomy 8, isochromosome 17q, and extra Ph chromosomes [32].

A bone marrow examination is mandatory if the molecular and cytogenetic studies are inconclusive such as in case of atypical CML or additional mutations, in such patients, it can reveal the specific cross-sectional histology pattern of disease progression. It additionally provides information on the level of hematopoietic suppression, which is important to decide on therapies mainly in the context of intensive treatment or stem cell transplantation [31].

2. c. Lactate Dehydrogenase (LDH):

Lactate dehydrogenase is an enzyme that serves as an indirect marker of tissue turnover and cell destruction. In CML, elevated LDH levels correlate with increased metabolic activity and disease burden. Higher LDH levels may indicate disease progression to the accelerated or blast phase and help in prognostication. Elevated LDH at diagnosis suggests a high leukemic cell turnover. A reduction in LDH levels following the initiation of TKI therapy indicates a favorable therapeutic response while, a persistently high or rising LDH levels may reflect disease progression or resistance to therapy [33].

Beyond its diagnostic value, LDH levels are increasingly being explored as a potential marker for treatment response stratification. Patients with persistently high LDH levels despite achieving hematological remission may require closer molecular monitoring. Elevated LDH is also associated with extramedullary disease manifestations, such as hepatosplenomegaly, which is commonly seen in advanced CML [33].

2. d. Karyotyping:

Karyotyping is an essential diagnostic and monitoring tool in CML which is used to detect numerical and structural chromosomal abnormalities. This technique is primarily used to detect the Philadelphia chromosome and additional chromosomal abnormalities. It helps in diagnosing the disease, predicting prognosis, and assessing response to therapy. Karyotyping is performed on bone marrow metaphase cells using Giemsa (G-banding) staining. During disease progression, clonal evolution may occur, leading to additional chromosomal abnormalities (ACAs) which are detectable by this technique. Some common ACAs include: Trisomy 8, Isochromosome 17q, Additional Philadelphia chromosome, etc[34].

2. e. Fluorescence In-Situ Hybridization (FISH) for the Philadelphia Chromosome:

The FISH technique is used in cytogenetics to detect the Philadelphia (Ph) chromosome, which is specific to CML. FISH has many advantages over conventional karyotyping in diagnosing CML. Utilizing FISH, *BCR::ABL1* fusion gene can, therefore, be viewed in interphase nuclei with rapidity and sensitivity [35].

FISH is of particular value in cases where standard cytogenetics fail due to poor metaphase yields or true cryptic translocations that are not visible on a standard karyotype. It detects minimal residual disease through the detection of low levels of Ph-positive cells following treatment, providing an early clue regarding disease relapse. This technique can be applied to peripheral blood samples, bone marrow aspirates, or even fixed tissue specimens, thus allowing great flexibility in testing[36].

The latest improvements in FISH technology have resulted in FISH and SKY, which greatly improve the ability to detect complex chromosomal aberrations associated with the progression of CML. These methods are a major advance for detecting further cytogenetic aberrations such as secondary translocations, deletions, or duplications, which may confer

resistance to the tyrosine kinase inhibitors[36]. FISH is essential for monitoring treatment response. FISH follow-up shows a lowering percentage of Ph-positive cells, hypothesizing good therapeutic response; otherwise, when Ph-positive cells are persistent under treatments, which suggests inadequate response or an emergence of resistance, thus requiring further molecular investigations[33].

2. f. Overview of Molecular Diagnostics in CML:

Molecular diagnostics justify their relevance in the diagnosis, monitoring, and assessment of CML treatment response. Polymerase chain reaction (PCR) is the primary molecular technique used to detect *BCR::ABL1* transcripts. Reverse transcriptase PCR (RT-PCR) is utilized for the qualitative detection of the *BCR::ABL1* fusion transcripts, and can detect them either in blood or bone marrow samples. RT-PCR has a very high sensitivity thus necessitating its use as a key technique for monitoring MRD[32].

For monitoring treatment response in CML, quantitative Real time PCR (qPCR) is considered the gold standard due to the possibility of very precise quantification of *BCR::ABL1* transcript levels. A major molecular response (MMR) is defined as 3-log reductions of *BCR::ABL1* transcripts from the baseline, while deep molecular response (DMR) is defined as undetectability or extremely low transcript levels below 4.5 log reductions of *BCR::ABL1* transcripts from the baseline. Regular qPCR monitoring gives an insight into therapeutic efficacy by detecting early signs of relapse.

NGS is another molecular technique which is increasingly being used to detect additional mutations responsible for resistance towards TKIs, thus helping to guide personalized treatment strategies[37]. With the development of these high-throughput sequencing techniques, the novel genetic alterations associated with CML progression have been characterized, which enables the refinement of risk stratification models. In addition, digital droplet PCR has emerged as a highly sensitive technique to detect low-level mutations and MRD while enhancing the ability to track disease evolution[32].

Mutation analysis of the *ABL1* kinase domain is critical for CML patients in whom first and second-generation TKIs became ineffective. Mutations like T315I are associated with resistance to 1st and 2nd generation TKIs, and options like ponatinib or allogeneic stem cell transplant would be considered. Only with comprehensive molecular profiling can more informed decisions be made regarding therapies that would optimize patient outcomes[37].

3. Utility of Commonly Utilized Molecular Diagnostic tests in CML:

3 (a): Qualitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) for *BCR::ABL1* testing

Molecular tests play a key role in diagnosing and managing CML. To establish the diagnosis of CML, qualitative reverse transcription PCR is very useful. RT-PCR measures *BCR::ABL1* mRNA levels compared to control genes like *ABL1, GUSB, or BCR*, which helps check sample quality. Unlike quantitative real time PCR (qRT-PCR), which measures transcript levels over time, qualitative RT-PCR confirms the presence or absence of the fusion gene and helps to determine the *BCR::ABL1* transcript to be measured during follow up. Various *BCR::ABL1* fusion transcripts result from differences in the breakpoint within the *BCR* gene, giving rise to distinct fusion protein isoforms:

Table 9: Types of *BCR::ABL1* Transcripts Detected by RT-PCR[38]

Fusion Transcript	Protein Product	Frequency in CML
e13a2 (b2a2)	p210 *BCR::ABL1*	~40-50%
e14a2 (b3a2)	p210 *BCR::ABL1*	~50-60%
e13a2 + e14a2	Both p210 variants	~10-15%
e1a2	p190 *BCR::ABL1*	Rare in CML, common in Ph+ ALL
e19a2	p230 *BCR::ABL1*	Rare, associated with neutrophilic CML (CML-N)

3 (b): Quantitative Real-Time PCR (qRT-PCR) for *BCR::ABL1* testing

Quantitative Reverse Transcription Real-time PCR (qRT-PCR) is the gold standard for monitoring molecular MRD in patients with CML. It quantifies *BCR::ABL1* transcript levels over time, allowing precise assessment of treatment response and early detection of relapse.

The principle of qRT-PCR involves three main aspects:

1.**Reverse Transcription (RT):** Converts *BCR::ABL1* mRNA into complementary DNA (cDNA).

2.**Quantitative PCR (qPCR):** Uses fluorescent probes to amplify and quantify cDNA, measuring transcript levels in real-time.

3.**Normalization:** *BCR::ABL1* transcript levels are compared to a control gene (e.g., *ABL1, GUSB, B2M, BCR*) to standardize results.

Table 10: Summary of Key Equipment & Reagents required for qRT-PCR *BCR::ABL1* quantitative testing [39]

Equipment Type	Example Models
RNA Extraction System	Extracts total RNA from peripheral blood or bone marrow.
Thermal Cycler (RT-PCR)	Converts extracted RNA into complementary DNA (cDNA) using reverse transcriptase enzyme.
Real-Time PCR Machine	Performs quantitative real-time PCR using fluorescent TaqMan probes or SYBR Green.
qPCR Plates/Tubes	96-well or 384-well PCR plates or 0.2 mL PCR tubes (low-binding, RNAse-free).
Pipettes & Tips	RNAse-free, sterile filtered pipette tips (to prevent contamination).
Fluorescent Probes	Probes for *BCR::ABL1* fusion detection.
Analysis Software	Analyzes *BCR::ABL1* transcript levels relative to control genes (e.g., ABL1, GUSB).
Reference Standards	WHO *BCR::ABL1* RNA Reference Standard (for normalization).
Refrigerated Microcentrifuge	RNA precipitation
-80°C Freezer & -20°C Freezer	long-term RNA & cDNA storage
Vortex Mixer	Sample homogenization
Autoclave & Sterile Work Area	Prevent cross-contamination

qRT-PCR finds a wide range of application in CML including: baseline molecular assessment, treatment monitoring, early detection of resistance or relapse and even providing therapeutic markers guiding TKI discontinuation (Treatment-Free Remission, TFR).

Laboratories report qRT-PCR *BCR::ABL1* results using the International Scale (IS) to make sure the results are reproducible universally. Doctors look at important molecular milestones to make treatment choices and see if patients can stop treatment while staying healthy (TFR). These milestones include early molecular response (EMR) major molecular response (MMR; ≤0.1% *BCR::ABL1* IS), and deep molecular response (DMR; ≤0.01% *BCR::ABL1* IS)[39].

3. b. Timing of Test as per international guidelines:

Molecular testing for chronic myeloid leukemia is carefully scheduled at different time points during diagnosis and treatment, as per guidelines from reputed organizations like the European Leukemia Net (ELN) and the European Treatment and Outcome Study (EUTOS). The timing and regularity of these tests are crucial for accurately diagnosing the disease, keeping track of how well treatment is working, spotting any resistance, and determining if a patient is eligible for treatment-free remission.

Table 11: Recommended Timing of Molecular Tests in Chronic Myeloid Leukemia (CML) as per Current Guidelines

Clinical Stage	Recommended Molecular Test and Timing
At Diagnosis	Qualitative RT-PCR to detect *BCR-ABL1* fusion gene and identify transcript variant (e13a2/e14a2 or atypical forms) [39]
First year of TKI therapy	Quantitative RT-qPCR every 3 months to monitor molecular milestones: ≤10% IS at 3 months (EMR), ≤1% IS at 6 months, and ≤0.1% IS (MMR) by 12 months [40]
After achieving stable MMR/DMR	Quantitative RT-qPCR every 6 months to ensure ongoing molecular response maintenance [40]
Before Treatment-Free Remission (TFR)	Confirm deep molecular response (MR4 or deeper) sustained for ≥2 years before discontinuing TKI [39]
After TKI discontinuation (TFR monitoring)	Quantitative RT-qPCR monthly for the first 6 months, then every 2-3 months up to 18 months; long-term follow-up every 3-6 months to detect molecular relapse [39]
In case of treatment failure or loss of MMR	Immediate *BCR-ABL1* kinase domain mutation analysis to detect TKI resistance mutations; use of Sanger sequencing or NGS based on sensitivity requirements [39][41]

At diagnosis, molecular testing begins with a qualitative RT-PCR to confirm the presence of the *BCR::ABL1* fusion gene, which is the defining feature of CML. Additionally, this test identifies the specific transcript variant (major, minor, micro), which is crucial for selecting the appropriate quantitative monitoring strategy. Cases expressing atypical *BCR::ABL1* variants require further characterization to ensure accurate follow-up testing [39]. Identifying the transcript type before therapy also helps avoid false-negative results and establishes a reliable baseline for subsequent molecular assessments.

Once a patient starts on tyrosine kinase inhibitor therapy, regular quantitative molecular testing kicks in to check how well they're responding. The ELN guidelines suggest that during the first year of treatment, quantitative RT-qPCR should be done every three months. These tests are essential for tracking whether the patient is achieving key molecular targets. At the three-month mark, achieving an early molecular response with *BCR::ABL1* transcript levels at or below 10% on the International Scale (IS) is a strong indicator of positive long-term outcomes. Monitoring continues at six months to see if *BCR::ABL1* levels fall to 1% IS or lower, and by the twelve-month point, patients should ideally reach a major molecular response, which is defined as *BCR::ABL1* levels at or below 0.1% IS[40]. If these milestones aren't met, it could indicate a less-than-optimal response or the development of resistance, which would require further assessment and possibly a change in treatment.

Once patients reach and maintain a significant molecular response, or even deeper levels like MR4 (*BCR-ABL1* ≤0.01% IS) or MR4.5, they can cut back on how often they get tested. At this stage, the guidelines suggest doing molecular tests every six months. This less frequent testing is seen as enough to keep the disease under control while avoiding unnecessary procedures. However, if there is any unexpected rise in *BCR-ABL1* transcript levels during this time, it's crucial to reassess the situation right away to check for any potential loss of response or disease progression[40].

For patients who manage to maintain a stable deep molecular response for at least two years, stopping TKI therapy to pursue treatment-free remission can be a viable option. However, it's important to note that the risk of molecular relapse is highest right after treatment is halted, so a more rigorous molecular monitoring schedule is crucial. According to the ELN guidelines, molecular testing should be done monthly for the first six months after discontinuing TKI therapy. After that, testing should continue every two to three months for at least the next twelve to eighteen months to quickly catch any signs of molecular relapse[39]. If there is a sustained loss of MMR or an increase in *BCR::ABL1* levels, it is necessary to restart TKI therapy to prevent a hematological relapse. When molecular testing shows rising *BCR::ABL1* transcript levels or a complete loss of the previously achieved response, testing for *BCR::ABL1* kinase domain mutations becomes essential.

This testing helps identify point mutations in the kinase domain of the *BCR::ABL1* gene, which could lead to resistance against certain TKIs. Guidelines suggest conducting mutation analysis at these critical junctures to help determine the best alternative therapies. While Sanger sequencing has been the traditional method for this, next-generation sequencing (NGS) is gaining popularity due to its superior sensitivity and ability to detect low-level mutations that could impact clinical decisions[39][41].

Sticking to these recommended testing intervals is crucial for catching any less-than-ideal responses early on, spotting resistance mutations, and making smart choices about therapy changes or safe discontinuation. Regular molecular testing, along with standardized reporting on the International Scale, has played a significant role in enhancing the survival rates and quality of life for CML patients during the TKI era[39][40][41].

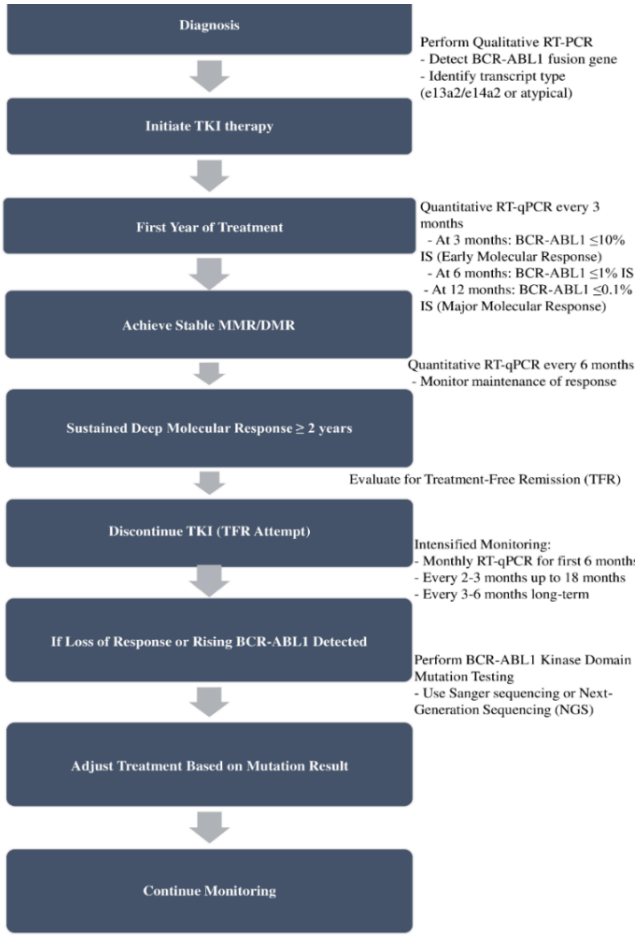

Diagnosis
Perform Qualitative RT-PCR
- Detect BCR-ABL1 fusion gene
- Identify transcript type
(e13a2/e14a2 or atypical)
Initiate TKI therapy
First Year of Treatment
Quantitative RT-qPCR every 3 months
- At 3 months: BCR-ABL1 ≤10% IS (Early Molecular Response)
- At 6 months: BCR-ABL1 ≤1% IS
- At 12 months: BCR-ABL1 ≤0.1% IS (Major Molecular Response)
Achieve Stable MMR/DMR
Quantitative RT-qPCR every 6 months
- Monitor maintenance of response
Sustained Deep Molecular Response ≥ 2 years
Evaluate for Treatment-Free Remission (TFR)
Discontinue TKI (TFR Attempt)
Intensified Monitoring:
- Monthly RT-qPCR for first 6 months
- Every 2-3 months up to 18 months
- Every 3-6 months long-term
If Loss of Response or Rising BCR-ABL1 Detected
Perform BCR-ABL1 Kinase Domain Mutation Testing
- Use Sanger sequencing or Next-Generation Sequencing (NGS)
Adjust Treatment Based on Mutation Result
Continue Monitoring

Figure 10: Molecular Testing Timeline in Chronic Myeloid Leukemia (CML)

In short, the suggested timeline for molecular tests in CML offers a well-organized and research-backed way to manage the disease effectively. Getting tested on time helps with accurate diagnoses, assessing the trend of therapeutic response, spotting any resistance early on, and keeping a close eye during attempts at treatment-free remission (TFR). Sticking to these timelines has played a huge role in the impressive long-term survival rates and improved quality of life for CML patients during the TKI therapy era.

3. c. qRT-PCR vs Other Molecular Techniques in CML MRD

Managing chronic myeloid leukemia (CML) leans heavily on advanced molecular diagnostic techniques, especially RT-PCR and qPCR. While both methods are rooted in PCR technology and focus on amplifying nucleic acids, they have distinct goals, approaches, sensitivities, and roles in the clinical landscape of CML.

A key advantage of qPCR over RT-PCR is its capacity to provide standardized results on the International Scale (IS). This standardization was created to ensure consistency across laboratories, enabling reliable comparisons of *BCR-ABL1* transcript levels worldwide [40].

The quantitative aspect of qPCR is essential for identifying specific molecular milestones, such as:

- Early Molecular Response (EMR): *BCR-ABL1* ≤10% IS at 3 months
- Major Molecular Response (MMR): *BCR-ABL1* ≤0.1% IS

- Deep Molecular Response (DMR): *BCR-ABL1* ≤0.01% IS or deeper

Hitting these milestones at specific times is linked to better long-term outcomes, like improved survival rates and the possibility of being eligible for Treatment-Free Remission (TFR)[39].

When it comes to sensitivity, qPCR really stands out compared to RT-PCR. While RT-PCR can effectively show whether *BCR-ABL1* transcripts are present, qPCR takes it a step further by detecting transcript levels that are as low as a 4 to 5-log reduction from the standardized baseline. This heightened sensitivity is crucial for spotting minimal residual disease (MRD) and helps guide clinical decisions, such as whether to continue therapy, adjust doses, or even stop treatment in cases of sustained deep molecular response (DMR)[40].

That said, qPCR does have its drawbacks. Its accuracy hinges on thorough assay validation, the careful selection of internal control genes (usually *ABL1 or GUSB*), and the creation of lab-specific conversion factors (CFs) to align with the International Scale[40]. Plus, external quality assurance (EQA) programs are vital for ensuring consistency across different labs[39].

To sum it up, qualitative RT-PCR serves mainly as a diagnostic tool, crucial for confirming the presence and type of *BCR-ABL1* fusion transcript at the initial diagnosis of CML. It gives a straightforward yes or no answer without detailing the disease burden. In contrast, qPCR is a quantitative, dynamic tool that's used throughout treatment to track disease progression, assess treatment response, and detect minimal residual disease with impressive sensitivity and accuracy. Both methods work hand in hand in managing CML, with RT-PCR laying the groundwork at diagnosis and qPCR steering therapeutic choices as the disease evolves.

MRD in CML refers to the detection and quantification of *BCR::ABL1* fusion transcripts using quantitative real-time PCR (qRT-PCR) after treatment with Tyrosine Kinase Inhibitors (TKIs). Molecular MRD monitoring helps assess treatment response, predict relapse, and guide treatment-free remission (TFR) strategies. Understanding MRD analysis is crucial for managing CML. The *BCR::ABL1* fusion gene is the molecular signature of CML, and measuring it with advanced techniques is what MRD assessment is all about. Right now, the gold standard test for CML-MRD monitoring real-time quantitative reverse transcription PCR, thanks to its impressive sensitivity and specificity[41].

In practice, we look for specific molecular milestones to evaluate how well the treatment is working. An Early Molecular Response (EMR) is noted when *BCR::ABL1* transcript levels drop to 10% IS or lower within three months, signaling a positive early response. A Major Molecular Response (MMR) is reached when *BCR::ABL1* levels are at or below 0.1% IS, along with more than 10,000 *ABL1* control gene copies. This is a vital therapeutic goal, as achieving MMR greatly lowers the risk of disease progression and serves as a key marker of effective treatment [40].

When patients achieve deeper levels of response, they fall into a category known as Deep Molecular Response (DMR). This classification is further divided based on how much the *BCR::ABL1* transcript has been reduced and the number of *ABL1* copies present. For instance, DMR-4 is identified when *BCR::ABL1* transcript levels are at 0.01% IS or lower, with *ABL1* copies ranging from 10,000 to 31,999. If the transcript levels drop to 0.0032% IS or lower and the *ABL1* copies are between 32,000 and 99,999, that's classified as DMR-4.5.

To reach DMR-5, the transcript level must be 0.001% IS or lower, with at least 100,000 *ABL1* copies. These deeper molecular responses correspond to reductions of 4-log, 4.5-log, and 5-log, respectively, when compared to the standardized baseline [41].

Table 12: qRT-PCR vs Other Molecular Techniques

Method	**Sensitivity**	**Use Case**	**Limitation**
Conventional Karyotyping	~5%	Detects Philadelphia chromosome	Cannot detect MRD
FISH (Fluorescence In Situ Hybridization)	~1%	Detects *BCR::ABL1* fusion in interphase nuclei	Limited sensitivity
Qualitative RT-PCR	~0.01%	Confirms presence of *BCR::ABL1*	Cannot quantify transcript levels
Quantitative RT-PCR (qRT-PCR)	~0.001%	Monitors MRD and TKI response	May not detect rare *BCR::ABL1* variants
Next-Generation Sequencing (NGS)	~0.0001%	Detects TKI resistance mutations (T315I, E255K)	Expensive, not routine for MRD

The quality of the sample is crucial for accurately interpreting MRD. If the control gene (*ABL1*) copy number drops below 10,000, the results are considered inconclusive, and a repeat sample is necessary due to reliability concerns. Moreover, any *BCR::ABL1* transcript level exceeding 0.01% IS, even with sufficient *ABL1* copies, indicates that there is no DMR [41]. Keeping an eye on MRD is essential not just for gauging the ongoing response but also for determining if TKI therapy can be stopped. Patients who maintain deep molecular responses, especially DMR-4.5 or deeper, for at least two years might be candidates for treatment-free remission. After stopping TKI treatment, MRD is monitored closely to catch any signs of molecular relapse, typically marked by the loss of MMR[40].

Newer technologies like digital PCR (dPCR) are making waves with their improved sensitivity and ability to provide absolute quantification, which enhances the detection of low-level MRD. dPCR can pick up *BCR::ABL1* transcripts even when RT-qPCR results show nothing, offering a more accurate assessment of deep molecular response and boosting the chances of predicting a successful TFR[41].

MRD analysis using advanced molecular techniques plays a vital role in effectively managing CML. It helps inform treatment choices, enables early identification of any treatment failures, and facilitates the safe stopping of therapy, all of which contribute to better outcomes for patients.

3. e. Importance of IS value:

The International Scale (IS) for measuring *BCR::ABL1* transcripts is essential for effectively monitoring CML therapy. It was created to tackle the inconsistencies that arise from different laboratories and methods when reporting *BCR::ABL1* levels. Before the IS was established, results from qRT-PCR tests varied widely due to differences in reagents, protocols, and

calibration methods, which made it tough to interpret results consistently and make informed clinical decisions [40].

With the implementation of IS, *BCR::ABL1* levels can be expressed as a percentage compared to a standardized baseline set during the International Randomized Study of Interferon and STI571 (IRIS) trial. On this scale, 100% IS represents the median baseline *BCR::ABL1* level for newly diagnosed CML patients, while specific molecular response levels are indicated by log reductions from this baseline. For instance, a *BCR::ABL1* level of ≤0.1% IS indicates a Major Molecular Response (MMR or MR3), and levels of ≤0.01% IS signify a Deep Molecular Response (DMR or MR4) [40].

The IS is vital because it guarantees that results are standardized and comparable across laboratories worldwide, which has a direct effect on treatment decisions. Reaching specific IS-defined milestones like EMR (≤10% IS at 3 months), MMR, or DMR is closely linked to better prognosis and helps shape treatment strategies, including the possibility of treatment-free remission (TFR).

Converting raw qPCR data into IS values requires using specific conversion factors (CFs) that are unique to each laboratory. These CFs can be determined through sample exchanges with reference labs, by using secondary reference panels that are calibrated to WHO standards, or through validated commercial kits. The European Treatment and Outcome Study (EUTOS) has shown that secondary reference panels are effective in validating CFs over time, which helps maintain consistency and quality control in MRD reporting[41].

Moreover, accurately assessing DMR is heavily dependent on the sensitivity and reproducibility of IS-standardized assays. For example, achieving MR4.5 and MR5 levels necessitates not just precise quantification of low-level *BCR::ABL1* transcripts but also ensuring that control gene copy numbers are appropriate (like *ABL1* needing to be ≥32,000 copies for MR4.5) to guarantee reliable interpretation, following standardized guidelines[39].

Not adopting IS-standardized reporting can lead to misinterpretations of molecular responses, which may result in misguided clinical decisions. Therefore, the IS value is more than just a reporting tool; it's a vital parameter that can significantly impact long-term patient outcomes, eligibility for treatment-free remission, and overall management of CML.

1. CML MRD testing by Quantitative qPCR:

Quantitative PCR (qPCR) remains the keystone in diagnosis, monitoring, and treatment evaluation among chronic myeloid leukemia. The *BCR::ABL1* fusion gene, resulting from the Philadelphia chromosome t(9;22), is the defining feature of CML and the primary target for treatment. The highly sensitive qPCR enables minimal residual disease detection, whereas the less sensitive varieties, such as conventional cytogenetic analysis and FISH, cannot. Among its major applications is the monitoring of treatment response in patients on tyrosine kinase inhibitors, including Imatinib, Dasatinib, and Nilotinib. qPCR helps measure the *BCR::ABL1* transcript levels in peripheral blood or bone marrow samples, thereby assessing the effectiveness of therapy and tracking molecular responses, including major molecular response (MMR), which means *BCR::ABL1* levels of 0.1% or lower on the International Scale, and deep molecular response, including MR4 (≤0.01%) and MR4.5 (≤0.0032%). qPCR has also been of great significance in the earliest detection of resistance to TKIs or relapse since an increase in *BCR::ABL1* level usually indicates diminishing response and the

necessity for treatment alteration. It is also an important tool for assessing minimal residual disease, detecting low levels of leukemia cells when cytogenetic tests might provide no abnormality results, thus forming the basis for guiding decisions as to the continuation or discontinuation of treatment in deep remission. Early molecular response, defined as *BCR::ABL1* level below 10% within the first three months, predicting better long-term outcomes and the unfulfilled molecular milestone-to-second-or-third-generation TKIs switch confers prognostic significance on qPCR. Its high sensitivity and specificity and its quantitative nature render qPCR an invaluable technique in the personalized management of CML.

4. a. Principle:

Quantitative PCR is also known as real-time PCR. This is a versatile method that can both amplify and quantify the amount of a specific DNA sequence present in a sample. In a conventional PCR reaction, the amplified product is detected only at the end of the reaction, while qPCR allows for the real-time monitoring of the DNA synthesis through different fluorescence systems used for detection. PCR is based on three well-established principles: denaturation, annealing, and extension, where with each successive cycle, the DNA polymerase enzyme is amplifying the target sequence [42]. The one aspect that distinguishes qPCR from regular PCR would be the addition into the PCR, fluorescent dyes or probes, whether that be SYBR Green or TaqMan that will attach to the DNA and give off fluorescence in direct correspondence with the amount of amplified DNA.

The fluorescence signal is measured during each cycle, enabling the calculation of the Ct value as the cycle number at which the fluorescence exceeds a set baseline threshold. It is inversely proportional to the initial amount of DNA in the sample; that is, the smaller the Ct value, the greater the initial concentration of DNA. The key methodologies for qPCR are absolute quantification, where the target DNA is compared against a standard curve to establish exact copy numbers, and relative quantification whereby normalization against reference genes are necessary to account for variations in gene expression.

A breakthrough in qPCR is represented by reverse transcription quantitative PCR (RT-qPCR), a technique used to quantify RNA and transcribe it in complementary DNA by using reverse transcriptase. The wide use of this method consists of, but is not limited to, gene expression studies, viral load estimation, and disease diagnostics. qPCR has become the cornerstone in many fields, such as clinical diagnostics, forensic analysis, agricultural biotechnology, and environmental monitoring because of its high sensitivity, specificity, and rapid turnover time. It is especially conducive to the detection of infectious agents, genetic mutation identification, and studies of molecular pathways with unparalleled precision[43]. Addressing the very minute quantities of nucleic acids with high reproducibility is the unit that makes qPCR an important tool in modem molecular biology and biomedical research.

4. b. Sample Requirement:

In qPCR, a specific quantitative assay for the diagnosis and monitoring of CML, sample type is of supreme importance. In practice, peripheral blood and bone marrow aspirates form the common samples. Generally, peripheral blood is preferred because it is a less invasive method of collection and typically contains a sufficient representation of leukemic cells;

however, for cases requiring more in-depth molecular analysis, bone marrow aspirates are required.

The specimen should be collected in an EDTA or a heparinized tube in order to prevent coagulation. In RNA-based qPCR, particularly for *BCR-ABL1* fusion gene detection, total RNA must be extracted from mononuclear cells, using TRIzol reagent or column-based extraction kits. RNA of very high quality is expected, with its RNA integrity number above 7.0, which will guarantee reliable reverse transcription into cDNA[44]. The quantification of *BCR::ABL1* transcript levels by RT-qPCR is crucial for CML diagnosis, treatment response, and minimal residual disease monitoring.

An absolute quantification requires a standard curve with known copy numbers for the *BCR::ABL1* oncogene, whereas relative quantification compares *BCR::ABL1* expression to a reference gene, usually *ABL1 or GUSB*, to normalize the results. The qPCR assay should be very sensitive to detect *BCR::ABL1* transcripts with a 4.5-log reduction concerning TKI therapy, and this is important for defining molecular response levels in CML patients [42].

Samples should be processed promptly or stored at -80°C to preserve RNA integrity. It is necessary to minimize contamination, RNA degradation, and the presence of inhibitors to PCR to ensure reliable and reproducible results by qPCR. In clinical and research settings for the management of CML, standard RNA isolation methods, such as those recommended by the International Scale (IS) for the quantification of *BCR::ABL1*, must be followed.

4. c. RNA Extraction Technique and Quality Control:

Thorough RNA extraction and quality control form an essential part of a molecular diagnostic test and treatment monitoring for CML. Fiber those, the realization of gene expression quantification, as well as an optimized RNA extraction technique, represent a key step for BCR-ABL fusion transcript detection.

RNA Extraction Techniques:

Several RNA extraction techniques have been explored for CML studies, particularly for isolating RNA from blood or plasma samples. The two most commonly used approaches include phenol-chloroform extraction (e.g., TRIzol method) and column-based extraction kits.

a. TRIzol-Based Extraction:

The TRIzol-based RNA extraction method is widely used because it effectively isolates high-quality RNA[45]. The method relies on the phase separation using phenol and guanidine isothiocyanate to isolate RNA while discarding DNA and proteins. Possible drawbacks include insufficient removal of contaminating genomic DNA and the need for meticulous practices to prevent RNA degradation.

b. Column-Based Extraction Kits:

Several commercial RNA purification kits have been developed that promise a better yield and purity of the RNA recovered. For instance, GeneAll® RiboEx™ showed that minor protocol adjustments made it superior to TRIzol for extracting high-quality RNA from plasma samples. Compared to TRIzol, column extraction methods allow the process to be conducted more straightforwardly, thereby lowering the risk of contamination[46]. The

underlying principle of this technique is the selective adsorption of RNA to silica in the presence of chaotropic salts. The lysis buffer used in this method contains guanidinium salts to lyse cells and inactivate RNases. In high salt conditions, RNA binds to silica. This is followed by washing steps remove contaminants, after which finally the extracted RNA is eluted in RNase-free water or buffer.

c. Magnetic Bead-Based Extraction

This technique is based on the process of nucleic acid binding to magnetic beads coated with silica or polymer. Magnetic beads capture RNA in presence of chaotropic salts or binding buffers. Magnetic beads are then exposed to magnetic field which holds the beads during wash steps. Finally, RNA is eluted in low-salt buffer or water.

c. Direct Lysis Methods

In this method the cells are lysed in a buffer that stabilizes RNA and inhibits RNases. This is a crude method where the lysate can be directly used in RT PCR reactions. However, this is a simplified approach for rapid analysis when RNA purity is less critical.

Quality Control (QC) in RNA Extraction:

The quality control of extracted RNA is vital because it guarantees that any of the downstream applications are reliable, like real-time quantitative polymerase chain reaction RT-qPCR. Typically, some common QC parameters will include the following.

RNA yield and Purity: A spectrophotometer, such as a NanoDrop, can measure RNA concentration mainly by measuring absorbance values at 260/280 nm and 260/230 nm. A ratio close to 2.0 is generally considered a good indication of RNA purity. If the ratio is less than 2.0, it could mean that some proteins or phenol has contaminated the RNA preparation. A low 260/230 ratio typically indicates the presence of salt contamination [46].

RNA Integrity: RNA integrity is assessed using agarose gel electrophoresis or Bioanalyzer-based RNA Integrity Number (RIN) analysis. Highly intact RNA should exhibit clear ribosomal RNA bands without significant degradation[45].

Reverse Transcription and qPCR Efficiency: The extracted RNA is subjected to complementary DNA (cDNA) synthesis by reverse transcription in support of real-time PCR analysis. The degree of amplification and the reproductive ability of housekeeping genes (*GAPDH, ABL1*) is taken as an indicator of RNA quality[43]. Therefore, samples with good RNA integrity produce qPCR results with consistency and reliability, important for minimal residual disease detection in CML patients.

Optimization of RNA extraction techniques and the implementation of robust QC measures in CML diagnostics and treatment monitoring are essential. Both TRIzol-based and column-based methods offer distinct advantages. However, RNA purity and integrity always take precedence in any molecular analysis for reliable diagnosis and treatment.

4. d. Real-Time PCR Process:

Quantitative PCR (qPCR) for Chronic Myeloid Leukemia is the foremost molecular technique to detect and quantify the *BCR-ABL1* fusion gene due to the Philadelphia chromosome translocation (t9;22). The presence of a constitutively active tyrosine kinase

promotes the uncontrolled proliferation of myeloid cells [47]. qPCR, therefore, finds itself very much in use for CML diagnosis, treatment monitoring, and minimal residual disease detection due to its respectably high sensitivity and specificity.

The technique begins with obtaining peripheral blood or bone marrow samples from suspected or diagnosed patients with CML. Total RNA then is extracted using column-based methods or phenol-chloroform methods to get rid of contaminants and inhibitors that might interfere with the reaction [48]. Thereafter, the purity and concentration of RNA are assessed consecutively, for example, spectrophotometer or fluorometric quantification, to check whether the valuable input sample is suitable for use in downstream applications.

As qPCR detects gene expression, not genomic DNA, extracted RNA is reverse-transcribed into cDNA using a reverse transcriptase enzyme. This reaction has primers directed against the *BCR::ABL1* transcript and a reference gene, which is often *ABL1*, to act as an internal control for expression normalization with changes in sample input. The reverse transcription step ensures that all the RNA has been effectively converted into cDNA, which will then serve as a template for amplification in qPCR.

A master mix is made up of the cDNA template, specific primers for *BCR::ABL1* and *ABL1*, DNA polymerase, dNTPs, buffer, and a fluorescent dye or probe for real time detection. This procedure usually applies a TaqMan probe-based method for qPCR detection, characterized by high specificity. This approach uses a probe that is specific to a sequence and is labeled with a fluorophore and a quencher, which gives off fluorescence as the probe is degraded in a reaction thought to be progressing during amplification[48]. Alternatively, SYBR Green dye, which binds to double-stranded DNA, can be utilized; however, the amplification specificity has to be verified through melt curve analysis.

The qPCR reaction is performed in a thermal cycler: initial denaturation of DNA at a high temperature to separate the strands, followed by cycles of denaturation, annealing of the primers initiate the process, and extension occurs as the polymerase enzyme synthesizes complementary DNA strands. It amplifies the target sequence exponentially in each cycle, whereby fluorescence emission becomes a real-time indicator of DNA amplification and increases in proportion to the amount of newly synthesized DNA. To quantify *BCR::ABL1* transcript levels, the qPCR assay usually includes a standard curve that uses serial dilutions of a well-characterized positive control sample [47]. The cycle threshold value (Ct), at which fluorescence exceeds background signal, is recorded for both *BCR::ABL1* and the reference gene *ABL1*; comparison of Ct values made against the curve enables the quantification of the absolute amount of *BCR::ABL1* transcripts. Relative quantitation is used more often in the clinical setting, where expression of *BCR::ABL1* is normalized to levels of *ABL1* and expressed as a percentage on the International Scale (IS).

The quality controls offered during the process, from execution to completion, ensure the accuracy and reliability of the results. Non-template controls (NTCs) are incorporated to rule out possible contamination whereas positive controls assure the sensitivity of the assay [48]. After amplification, melt curve analysis is performed with SYBR Green to distinguish specific products from non-specific amplifications or primer-dimers. Triplicate assays are usually run in qPCR to ensure reproducibility, and the amplitude of detected signals assessed in qPCR can be used to compare variations in transcript levels in the samples tested. qPCR is one of the most crucial techniques in CML treatment monitoring, specifically for the patients

taking TKI therapy such as imatinib, dasatinib, or nilotinib. With the assistance of continuous monitoring over time, qPCR allows the assessment of the molecular response by watching for the decrease of *BCR-ABL1* transcript levels [47].

The identification of major molecular response (*BCR::ABL1* ≤ 0.1% on the IS) and *BCR::ABL1* transcript copy's decline during treatment demonstrates the success of therapy, whereas, on the other hand, resistant cases or relapses will come at some more definite levels, thus opening doors to the next level of intervention. qPCR being one of the most sensitive, rapid, and quantitative means assays not only enshrined itself as the gold standard for measuring molecular monitoring in CML but also guided important decisions such as identifying instances of treatment resistance.

4. e. Real Time PCR Analysis:

When examining qPCR results in Chronic Myeloid Leukemia, researchers look to spot and measure *BCR::ABL1* fusion gene transcripts. These transcripts act as a crucial molecular sign for diagnosing keeping an eye on treatment, and finding tiny traces of the disease. The first thing analysts do is check the cycle threshold (Ct) value. This value shows the cycle where fluorescence goes above the background threshold[47]. A Ct value that's lower points to more *BCR::ABL1* expression meaning the disease is more severe. On the flip side, a higher Ct value hints at fewer transcripts or possible recovery. In most cases, patients just diagnosed with CML have low Ct values because they have lots of leukemia cells. As treatment starts to work, patients' Ct values tend to go up as their *BCR::ABL1* transcript levels go down.

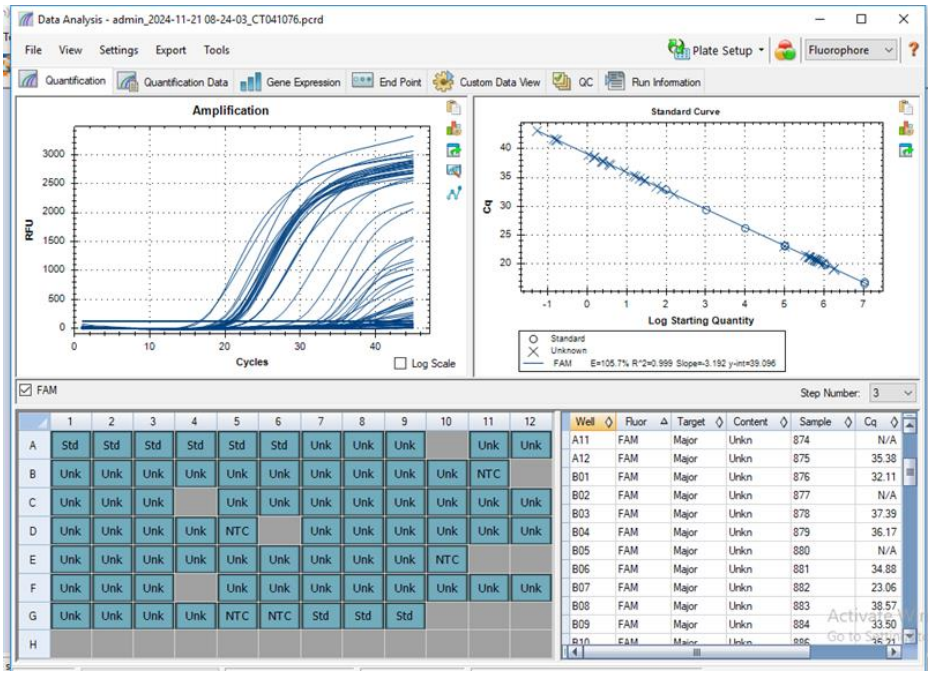


Figure 11: Amplification plot and standard curve generated using Bio-Rad CFX Maestro software. The amplification plot shows fluorescence intensity (RFU) versus cycle number for FAM-labeled targets. The standard curve demonstrates the linear relationship between Cq values and log starting quantity of standards, indicating PCR efficiency and R^2 value. The plate layout includes standards, unknown samples, and NTCs (No Template Controls) analyzed for Major *BCR-ABL1* transcript detection.

To get accurate numbers, labs often make a standard curve with known amounts of positive control samples. This curve shows how well the reaction works, which should be between 90-110% with a slope around -3.3. The R^2 value should be near 1.0 showing very precise

measurements. A good standard curve lets labs count how many *BCR-ABL1* transcripts are in a patient's sample by comparing their Ct values to the standards. But in everyday use, doctors look at relative amounts instead[48]. They compare *BCR-ABL1* levels to a housekeeping gene like *ABL1* to make sure all samples are treated the same. The final number is shown as a percentage on the International Scale (IS), which helps different labs share results that mean the same thing.

Doctors group the body's reaction to treatment into categories based on *BCR-ABL1* transcript amounts. A Complete Molecular Response (CMR) or Deep Molecular Response (DMR) happens when doctors can't find *BCR-ABL1* transcripts or find very few usually less than 0.01% on the IS. Getting a DMR, like MR4.0 (≤0.01% IS) or MR4.5 (≤0.0032% IS), means the cancer is less likely to come back. A Major Molecular Response (MMR) means *BCR-ABL1* levels are at or under 0.1% on the IS. This shows a thousand-fold drop from the start and is key to keeping the disease in check long-term[48]. An Early Molecular Response (EMR) means *BCR-ABL1* levels are ≤10% at three and six months. This often points to good treatment results. If a patient doesn't get an EMR, it might mean the treatment isn't working well. Doctors might need to watch the patient more or change the treatment. If a patient who had an MMR before now has *BCR-ABL1* levels above 0.1% IS, doctors call it a loss of molecular response. This could mean the treatment has stopped working, the patient isn't taking their medicine, or the disease has come back.

It is necessary to conduct additional molecular testing to evaluate resistance mechanisms when qPCR results indicate rising *BCR-ABL1* transcript levels despite continued therapy. Finding resistance-associated mutations like T315I, which result in insensitivity to specific tyrosine kinase inhibitors (TKIs) and call for a change in treatment approaches, requires mutation analysis of the *ABL1* kinase domain. Strict quality control procedures are used to preserve accuracy and dependability. These procedures include reference gene controls to guarantee RNA integrity, positive controls to confirm assay sensitivity, and no-template controls to rule out contamination[47]. Melt curve analysis is used to verify specificity by distinguishing true amplification from non-specific products when employing SYBR Green-based qPCR, and triplicate runs are carried out to improve reproducibility.

Frequent qPCR testing is necessary to monitor the course of the disease and evaluate treatment response. qPCR determines baseline *BCR-ABL1* transcript levels at diagnosis. For the first year of treatment, testing is done every three months to track early molecular response; after stable MMR is reached, testing is done every six months[48]. If there are indications of disease progression or loss of response, more frequent testing is necessary. Early treatment failure detection is made possible by longitudinal monitoring using qPCR, which directs prompt interventions like switching to second- or third-generation TKIs.

Clinicians can evaluate treatment effectiveness, identify resistance early, and decide on therapeutic changes with the help of the thorough analysis of qPCR results, which offers important insights into the molecular dynamics of CML. Since qPCR offers high sensitivity and accuracy in disease assessment, it continues to be the gold standard for molecular monitoring in CML thanks to the application of standardized methodologies, compliance with the International Scale and strict quality control measures.

4. f. Quality Control of Real-Time PCR:

For Chronic Myeloid Leukemia (CML), quality control (QC) in real-time PCR (qPCR) guarantees precise and repeatable *BCR-ABL1* transcript detection. It starts with RNA extraction, where fluorometric techniques like Qubit and spectrophotometry (260/280 nm ratio) are used to evaluate purity and integrity. Reliable reverse transcription (RT) requires high-quality RNA, which is confirmed by housekeeping genes like *ABL1* to identify any genomic DNA contamination and validate successful cDNA synthesis.

Positive controls confirm assay sensitivity during qPCR, while no-template controls (NTC) look for contamination. Reaction efficiency is evaluated using a standard curve made from serial dilutions of a known *BCR-ABL1* positive sample; this should be between 90 and 110 percent with an R2 value greater than 0.98. To ensure that differences in sample input or RT efficiency do not impact results, reference genes are utilized to normalize *BCR-ABL1* levels. Well-designed probes ensure precise binding to the BCR-ABL1 fusion transcript in probe-based assays like TaqMan, without cross-reacting with the normal ABL1 gene [42].

To guarantee consistent heating and fluorescence detection, instrument calibration is crucial, as are routine inspections of thermal cyclers. Melt curve analysis guarantees specificity in SYBR Green-based qPCR by identifying primer-dimer formations or non-specific products. Well-crafted probes guarantee accurate binding to the *BCR-ABL1* fusion transcript in probe-based assays such as TaqMan without exhibiting cross-reactivity with normal *ABL1[42]*.

Testing in triplicate reduces variability and improves the dependability of the results. To avoid problems like pipetting errors or reagent inconsistencies, the coefficient of variation (CV) between replicates must stay within allowable bounds. For worldwide comparability, results are standardized using the International Scale (IS), and accuracy is further confirmed by external quality assurance (EQA) programs[43].

By implementing these QC measures, qPCR remains a highly sensitive and reliable tool for diagnosing CML, monitoring treatment response, and detecting minimal residual disease (MRD), ensuring accurate patient management.

2. Assessing And Monitoring Tyrosine Kinase Inhibitor (TKI) Response in CML

The European LeukemiaNet (ELN) recommends that treatment response in CML be monitored using both conventional cytogenetics—specifically, chromosome banding analysis (CBA) of at least 20 metaphases from bone marrow cells—and real-time quantitative polymerase chain reaction (RT-qPCR). As previously stated, cytogenetic evaluations should be conducted at 3, 6, and 12 months until a complete cytogenetic response (CCyR) is achieved. After achieving a complete cytogenetic response (CCyR), chromosome banding analysis (CBA) can be substituted with fluorescence in situ hybridization (FISH) performed on a minimum of 200 nuclei from peripheral blood cells [49].

RT-qPCR should be performed every three months using buffy coat blood cells. ELN also states that when RT-qPCR methods are standardized and results are reported using the International Scale (IS) as *BCR::ABL1*%, this method alone may suffice for monitoring treatment response—not just after CCyR, but even from the outset—since RT-qPCR is more sensitive and does not require bone marrow sampling.

After achieving a major molecular response (MMR, defined as *BCR::ABL1* $\leq$ 0.1% IS), RT-qPCR testing can be conducted every 3 to 6 months, depending on factors such as baseline

risk, transcript levels, and their fluctuations. For patients who discontinue therapy following a stable, deep molecular response, monthly monitoring is advised for the first year, followed by testing at least every three months thereafter.

ELN categorizes responses into three clinical grades:

Optimal: The current therapy should be maintained, as the response suggests excellent outcomes and near-normal life expectancy.

Failure: A change in treatment is necessary, as the current response indicates a high risk of disease progression and mortality.

Warning: This intermediate category signals suboptimal response. Although better outcomes may be possible, there is insufficient evidence to recommend specific changes in therapy.

The response criteria for first-line imatinib treatment are supported by strong clinical evidence.

In contrast, response definitions for second-line treatments are provisional and primarily based on data from dasatinib and nilotinib.

Table 13: ELN definition of the response to TKIs, first-line

Time	Optimal	Warning	Failure
Baseline	NA	High risk or CCA/Ph +	NA
3 months	BCR/ABL1≤10 % or Ph + ≤35 %	BCR-ABL1 > 10 % or Ph + 36–95 %	Non CHR or Ph+ >95 %
6 months	BCR-ABL1≤1 % or Ph + 0 (CCyR)	BCR-ABL1 1–10 % or Ph + 1–35 % (PCyR)	BCR-ABL1 > 10 % or Ph + >35 %
12 months	BCR-ABL 1≤0.1 % (MMR)	BCR-ABL1 0.1–1 %	BCR-ABL1 > 1 % or Ph + ≥1%

Table 14: ELN definitions of the response to TKIs, second line

Time	Optimal	Warning	Failure
Baseline	NA	High risk, CHR never achieved, Loss of CHR, CyR never achieved	NA
3 months	BCR-ABL1≤10 % or Ph + <65 %	Ph + 65–95 %	No CHR BCR-ABL1 > 10 % Ph + >95 % New mutations
6 months	BCR-ABL1≤10 % or Ph + ≤35 % (MCyR)	Ph + 35–65 %	BCR-ABL1 > 10 % Ph + >65 % New mutations
12 months	BCR-ABL≤1 % or Ph + 0 (CCyR)	BCR-ABL1 1–10 % or Ph + 1–35 % (PCyR)	BCR-ABL1 > 10 % Ph + >35 % New mutations

NA - not applicable, CCyR - complete cytogenetic response, PCyR- partial cytogenetic response, MCyR- Major cytogenetic response, CHR- complete hematological response

Ref: TRUPCR® BCR-ABL1 Kit Version 2.0 (www.3bblackbio.com)

RESULTS

Our study involved a total of 61 patients who were diagnosed with CML. The demographic and haematological characteristics of the enrolled CML patients are summarized in Table 15. In our study group, 65.5% of the patients were males and 34.5% were females. The median age in our study group was 46 years. We divided the patients in the study into younger age group with cases ≤ 46 years age (29.5%) and older age group with cases >46 years age (70.5%). (Table 15).

We divided our study cases into two groups: untreated patients (UT) 15% (n=9/61) and treated patients (TP): those patients who received treatment with tyrosine kinase inhibitors (TKI) 85% (n=52/61). The treated patients were further subdivided into two groups: Treatment responders (TR) and Treatment non responders (TNR) based on those patients who responded optimally to TKI (TR) and those whose response was considered "warning" or "failure" as per ELN-CML guidelines. 40 patients out of 52 were grouped as TR (77%) while 12 patients were grouped as TNR (23%) (Figure 12).

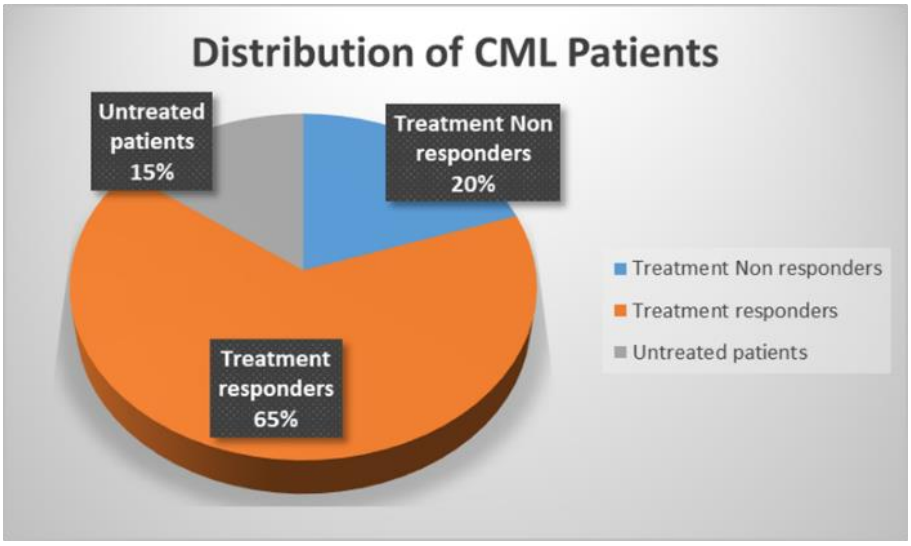


Figure 12: Distribution of CML patients as Untreated (UT), Treatment non-responders (TNRs), and Treatment responders (TRs)

Table 15: Clinical and hematological characteristics of CML patients

	N (%)	Groups		
CML Patients		**UT (%)**	**TNR (%)**	**TR (%)**
Total	61(100)	9(14.8)	12(19.7)	40(65.5)
Gender				
Male	40(65.5)	7(17.5)	5(12.5)	28(70)
Female	21(34.5)	2(9.5)	7(33.4)	12(57.1)
Age				
≤46	30(49.2)	5(16.7)	6(20)	19(63.3)
>46	31(50.8)	4(13)	6(19.3)	21(67.7)

RBC				
Within Normal RBC count	6(9.8)	0(0) [0]	2(33.4) [17%]	4(66.6) [10%]
Abnormal RBC count	55(90.2)	9(16.4) [100%]	10(18.2) [83%]	36(65.4) [90%]
WBC				
Within Normal WBC count	47(77)	1(2.1) [11%]	11(23.4) [92%]	35(74.5) [88%]
Abnormal WBC count	14(23)	8(57.1) [89%]	1(7.2)[8%]	5(35.7) [12%]
Platelet				
Normal platelet count	44(72.13)	4(9) [44%]	7(15.9) [58%]	33(54) [83%]
Abnormal platelet count	20(32.78)	5(25)[56%]	5(25) [42%]	10(50) [17%]
Hemoglobin (Hb)				
Within Normal Hb level	10(16.2)	1(10)[11%]	1(10) [8%]	8(80) [20%]
Abnormal Hb level	53(86.8))	8(15)[89%]	11(20.7) [92%]	32(60.3) [80%]
Hematocrit (HCT)				
Within Normal HCT level	14(23)	4(28.6) [44%]	2(14.3) [17%]	8(57.1) [20%]
Abnormal HCT level	47(77)	5(10.7) [56%]	10(21.3) [83%]	32(68) [80%]

UT- Untreated, TNR- Treatment Non Responders (MR 0, 1, 2), TR- Treatment Responders (MR 3, 4.5, 5)

All the CML patients in our study were positive for Philadelphia chromosome [t(9;22)] and the resulting *BCR::ABL1* fusion protein at diagnosis. Upon analyzing the hematological findings within our patients, we found that majority of the patients who didn't receive any prior therapy (UT), had abnormal hemoglobin levels (89%), RBC counts (100%) and leukocytosis (89%). We analyzed the patients who received TKI therapy and found that majority of these patients had anemia (mean Hb) and abnormal hematocrit levels (mean HCT). When we compared the hematological profiles of TR and TNR group we observed that treatment non responders presented more often with thrombocytopenia as compared to TR group. We also observed that total leukocyte count, total RBC count, Hb levels and hematocrit were not very useful in differentiating TR from TNR group. (Table 15).

All 9 UT patients underwent qualitative *BCR::ABL1* reverse transcriptase PCR analysis to determine the *BCR::ABL1* transcript type. All the patients (61) were determined to harbor *BCR::ABL1* Major (p210) transcript at diagnosis. None of our patients had minor or micro *BCR::ABL1* transcripts.

Following this we performed quantitative real time PCR (qPCR) based analysis for *BCR::ABL1* transcript to reveal the measurable residual disease status of our study group patients. We calculated the NCN % and subsequently determined the NCN (IS)% utilizing

the kit provided conversion factor (CF= 0.733). We detected a significantly higher number of *BCR::ABL1* major fusion copies among the UT group. We also observed higher *BCR::ABL1* major fusion copies in the TNR group while minimal copy numbers were detected in the TR groups. Thus, we observed a decreasing trend in average fusion copies, with the lowest average observed in the TR group **(Figure 13).**

Similarly, when comparing NCN% and IS values between different groups, we found UT group having the highest NCN and IS%, followed by TNR group. Minimum or undetected NCN and IS % was reported in the TR group. Accordingly, the average %NCN and % IS-NCN levels displayed a progressive decrease, indicating a move towards major molecular response, in the TR group when compared to the other two groups (UT and TNR groups) **(Figure 14).**

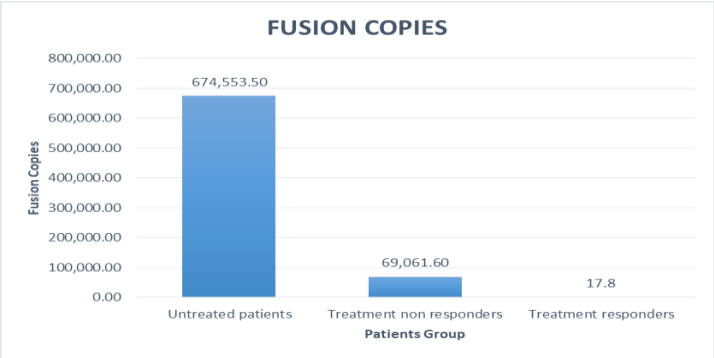


Figure 13: Fusion copies among different patient groups

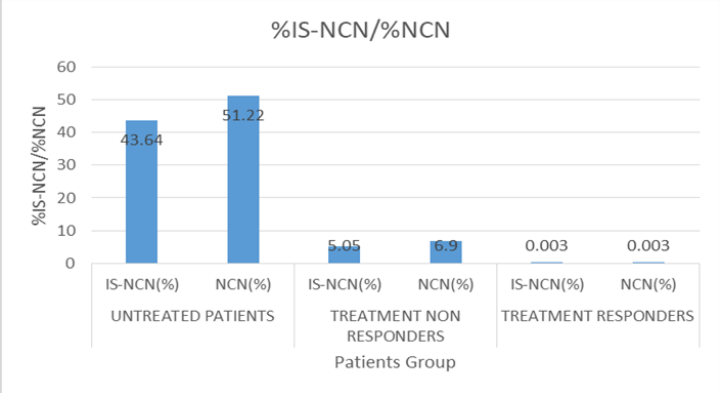


Figure 14: %IS-NCN and %NCN among different patient groups

Table 16: Correlation of hematological parameters among different patient groups

Haematological Parameters	**UT**	**TNR**	**TR**	**p-value**
RBC(g/dl)	3.35	3.65	3.75	0.17
WBC(x10^3cells/ul)	97.74	6.14	6.55	**0.0001**
Platelets (x10^3cells/ul)	243.11	195.6	161.68	1
Hb (g/dl)	10	11.3	11.3	0.16
HCT (%)	32.6	32.8	35.6	0.15

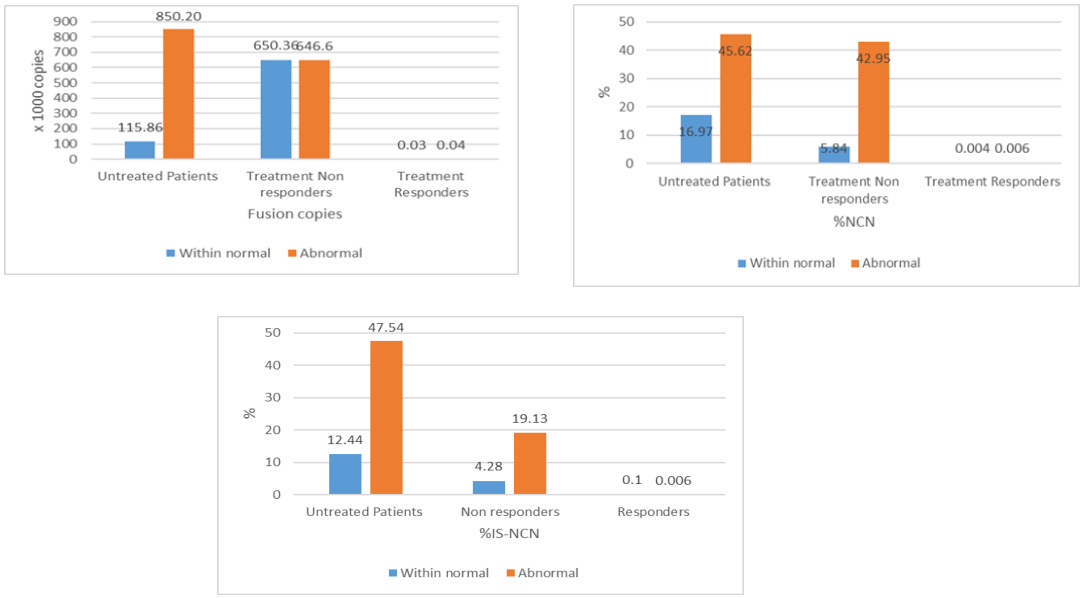


Figure 15: Correlation of fusion copies, % NCN levels and %IS-NCN of *MBCR-ABL1* with WBC count in UT, TNR and TR groups of CML patients

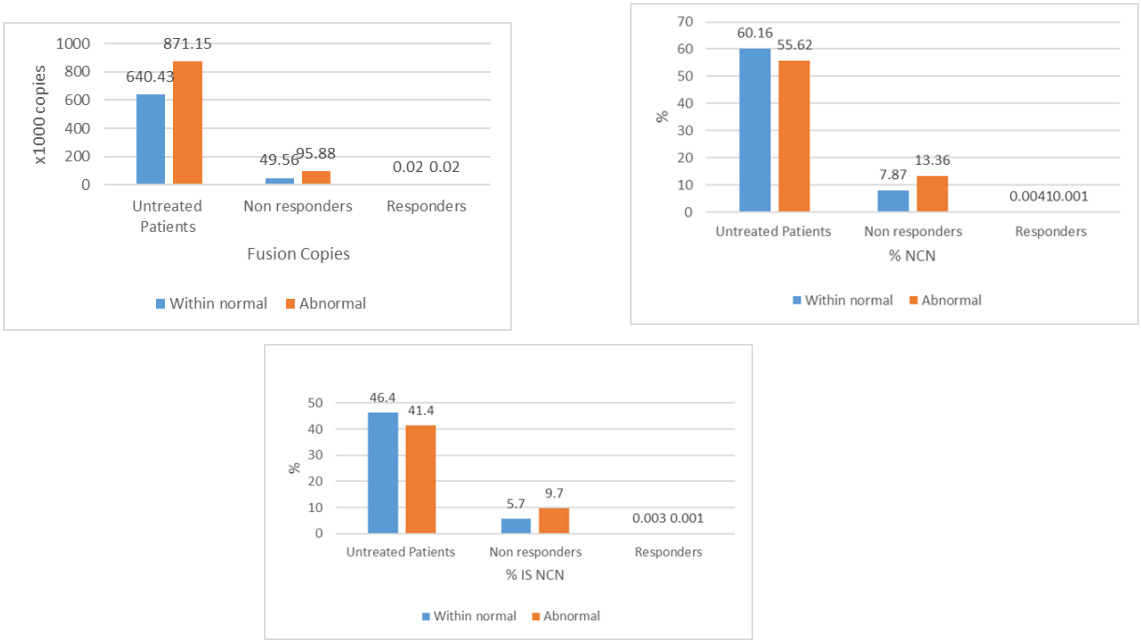


Figure 16: Correlation of fusion copies, % NCN levels and %IS-NCN of *MBCR-ABL1* with platelet count in UT, TNR and TR groups of CML patients

Correlation of *MBCR-ABL1* with various hematological parameters among UT, TNR and TR groups of CML patients

UT patients with higher expression of *BCR::ABL1* copies and higher NCN and NCN IS % consistently presented with abnormal WBC counts as compared to patients who received treatment with TKI. We also observed that TR patients consistently presented with lower NCN and IS% and also had lower incidence of abnormal leukocyte counts. On the other hand, CML patients with abnormal platelet counts had higher levels of % IS-NCN of *MBCR-ABL1* in UT and TNRs (Figure 12).

Utility of qPCR for determining therapeutic response among patients receiving TKI

Quantitative PCR (qPCR) is an efficient and reproducible technique for evaluating patient response in those given Tyrosine Kinase Inhibitors (TKIs) for disorders such as Chronic

Myeloid Leukemia (CML). Using qPCR to evaluate MRD is a means of detecting low numbers of leukemic cells, through quantification of *BCR-ABL1* fusion transcripts. This is especially useful as it can detect minimal residual disease in amounts that other methods would fail to identify. Tracking *BCR-ABL1* over time by qPCR is an objective modality for assessing reduction in *BCR-ABL1* transcripts that directly correlates to TKI therapy effectiveness.

qPCR is also used to detect early relapse. The ability of qPCR to detect rising levels of *BCR-ABL1* prior to any clinical symptoms of relapse can offer clinicians an early opportunity to intervene and alter clinical management - thus potentially affecting the patient outcome. Therefore, qPCR has great utility not only in response assessment, but also in early prediction of relapse. qPCR also allows for comparison between different TKIs and thus speeds the avenue to identifying the optimal drug for the individual patient, especially in the case of less-than-optimal initial TKI response. In all, qPCR is indispensable in guiding treatment decisions and improving long-term prognosis by providing precise, real-time data on disease response and relapse risk.

Type of TKI received-

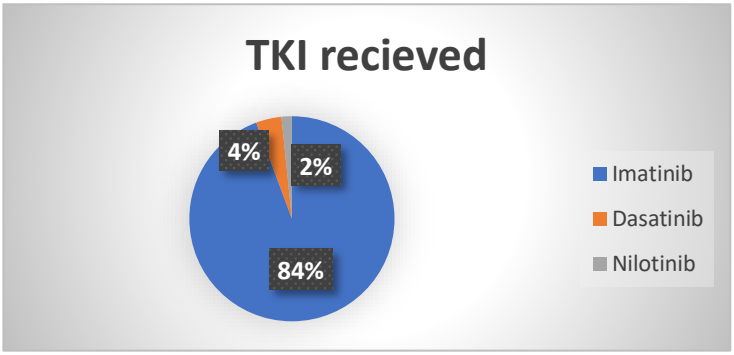


Figure 17: Distribution of tyrosine kinase inhibitors (TKIs) administered among patients in the TNR and TR groups. The majority received Imatinib (94%), followed by Dasatinib (4%) Nilotinib (2%)

- **Table 17: *BCR-ABL: ABL* Levels by RQ-PCR on Imatinib therapy.**

NCN –IS (%)	Number of patients at 6 months	Number of patients at 12 months	Number of patients > 12 months
>10%	07	02	04
<10-1%	02	0	01
<1-0.1%	0	0	11
<0.1-0.01%	0	0	09
<0.01-0.001%	0	0	08
<0.001%	0	0	17

- **Table 18: Analysis of MMR status of Study group**

Time point	Optimal	Warning	Failure
Baseline	0	01	03
3 months	0	01	03
6 months	0	0	03
12 months	0	0	02

>12 months	40	0	07

- **Table 19: History of Drug escalation/ Drug change**

	TNR	**TR**
Drug escalation	12	37
Drug change	0	03

- **Table 20: History of TKD mutation analysis**

	Number of patients
TKD mutation present	01
TKD mutation absent	02
Total number of patients tested	03

- **Table 21: MRD trend in the study group among patients receiving TKI.**

Therapy dynamics	**TR**	**TNR**
Ascending trend	01	03
Decreasing trend	22	05
Plateau	10	03
Irregular	07	01

- **Table 22: Analysis of treatment dynamics of TNR group:**

Never achieved MMR	9%
Loss of MMR	12%
Loss of CHR	79%

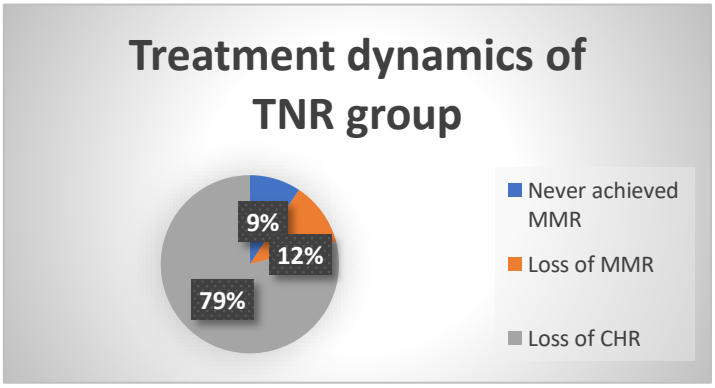


Figure18: Pie chart representing treatment dynamics in the TNR group. The majority (79%) has loss of CHR, while 12% experienced loss of MMR, and 9% each never achieved MMR or had a loss of MMR.

- **Table 23: Analysis of treatment dynamics of TR group**

Achieved MMR on 12 months	0%
Achieved MMR after 12 months	25%
Lost and again achieved MMR	0%
Achieved DMR	75%

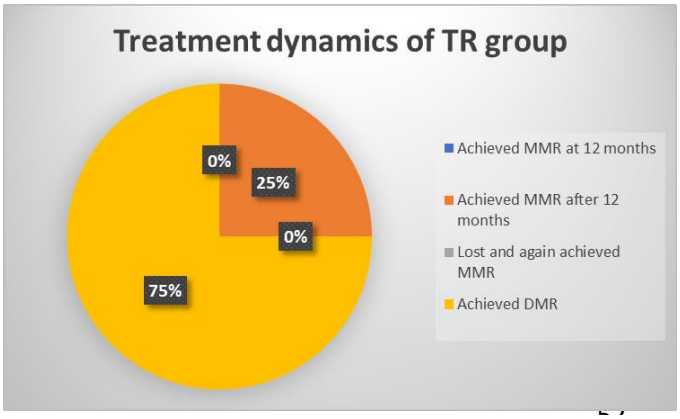


Figure 19: Pie chart illustrating treatment dynamics in the TR group. The majority (75%) achieved DMR, while 25% achieved MMR after 12 months. No patients achieved MMR at 12 months or lost and regained MMR.

DISCUSSION

Chronic Myeloid Leukemia (CML) often manifests with vague, systemic complaints such as persistent fatigue, general malaise, and unintended weight loss. On examination, splenomegaly is a common finding, and as the disease progresses, symptoms related to leukostasis—such as headaches, visual impairment, or priapism—may emerge. Given the overlap in clinical features, particularly leukocytosis and splenic enlargement, differential diagnosis should include a range of myeloproliferative and infectious conditions, warranting a detailed diagnostic workup. Classification of CML relies on identifying its progression through three phases—chronic, accelerated, and blast—based on hematologic parameters, bone marrow analysis, and clinical presentation.

Laboratory evaluation of CML typically reveals marked leukocytosis with a left shift in the myeloid lineage, accompanied by thrombocytosis and varying degrees of anemia. Bone marrow aspiration often demonstrates pronounced hypercellularity with an elevated granulocyte-to-erythroid (G:E) ratio and an expansion of myeloid precursors. A definitive diagnosis is established by identifying the Philadelphia chromosome (Ph), resulting from the t(9;22) (q34; q11) translocation, or its molecular counterpart, the *BCR::ABL1* fusion gene. Cytogenetic techniques such as conventional karyotyping and FISH are essential for detecting this chromosomal abnormality. In addition, molecular assays like quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR) enable sensitive quantification of *BCR::ABL1* transcript levels, facilitating both disease monitoring and assessment of therapeutic response. qPCR-based MRD is considered a cornerstone of modern CML management which is essential for tailoring therapy, assessing prognosis, and enabling potential treatment-free approaches.

In the present study, the median age of the study group was found to be 46 years. Similar studies have reported that the median age at diagnosis of CML in Asia and Africa is below 50 years, indicating a relatively lower age at onset in these regions[50]. Moreover, in current study, among 61 patients, 40 (65.5%) were males and 21 (34.5%) were females, indicating a male predominance in CML incidence. This observation aligns with previous findings by Amin et al (2021), Radhi et al (2023) and Kumar et al (2019)[26][5][51]. The male predominance may be attributed to the higher prevalence of haematological neoplasms in males, potentially influenced by genetic and hormonal disparities. In addition, a gender disparity might exist in our society due to which males regularly can afford diagnostic investigations as compared to females.

The comparison across untreated, treatment non-responders, and treatment responder groups reveals distinct averages for various haematological parameters. Specifically, the untreated group exhibits an average RBC count of 3.35 x 103 cells/µl, while the treatment non-responders and responder groups show averages of 3.65 x 103 cells/µl and 3.75 x 103 cells/µl, respectively. WBC counts are measured at 97.74 x 103 cells/µl, 6.14 x 103 cells/µl, and 6.55 x 103 cells/µl for UT, TNR and TR groups, respectively. Platelet counts follow a similar trend with values of 243.11 x 103 cells/µl, 195.6 x 103 cells/µl, and 161.68 x 103 cells/µl in UT, TNR and TR groups, respectively. Haemoglobin levels are recorded at 10 g/dl, 11.3 g/dl, and 11.3 g/dl, while HCT levels are measured at 32.6%, 32.8%, and 35.6% in UT, TNR and TR groups, respectively. Statistical analysis indicates significant differences in WBC count (p=0.0001) between treatment TNRs and TRs, while platelet count (p=1) RBC

count (p=0.17), Hb level (p=0.16), and HCT level (p=0.15) do not exhibit significant variations among the groups.

Analysing the haematological parameters like RBCs, WBCs, platelets, haemoglobin count, and haematocrit count, this study aligns with previous research. Specifically, studies by Pinto et al (2021), Ting et al (2017), Karaosmanoglu et al (2023) and Kumar et al (2019)[52][46][24][5]found no significant difference in median RBC levels between patient groups. Regarding WBC levels, present results correspond with those of Amin et al (2021), Arun et al (2017), Karaosmanoglu et al (2023), Kumar et al (2019) and Bhatti et al (2012)[26][53][24][5][54], which showed a high significant difference between patients. However, concerning haemoglobin level, current results contradict those of Kumar et al. (2019), Bhatti et al (2012) and Granatowicz et al (2015)[5][54][55] which demonstrated a significant difference between patient and control groups. Lastly, present study aligns with previous research by Pinto et al (2021), Ting et al (2017), Karaosmanoglu et al (2023) and Kumar et al (2019)[52][46][24][5] indicating no significant difference in haematocrit levels between patient groups. The UT group shows an average LDH level of 3139.4 U/L, while LDH data for the TNR and TR groups was unavailable. This also points to the fact that haematological and biochemical assays (e.g. LDH) are not sufficient to reflect the MRD status in haematological neoplasms, and thus requires cytogenetic and molecular MRD assessments to manage these patients better.

On assessing %NCN and %IS-NCN counts among three patient groups (UT, TR and TNR), untreated patients (UT) exhibit the highest levels of both measures, with averages of 57.65 and 36.35, respectively. This is followed by treatment non-responders and treatment responders, with significantly lower averages of 9.7/7.1 and 0.0038/0.0032, respectively. These findings strongly indicate the impact of treatment response on %NCN and %IS-NCN levels, showcasing an inverse correlation; as treatment response decreases, the levels of %NCN and %IS-NCN increase. Hence, a lower treatment response corresponds to higher %NCN and %IS-NCN levels, underscoring the importance of treatment efficacy in patient management. Similar findings have been reported by Jethva et al (2022) and Son NT et al (2021) [56][57] who analysed 50 Indian and 172 Vietnamese CML patients respectively. This further establishes the importance of quantitative PCR tests in segregating CML patients according to their therapeutic response.

As noted above, this study also demonstrated that Indian generic imatinib mesylate (IM) is a viable, cost-effective, clinically advantageous option for CML-CP with treatment outcomes comparable to the original branded molecule, Gleevec. This small cohort of study participants also demonstrates how central molecular monitoring with real-time quantitative PCR (RQ-PCR) can be influential in determining the presence of minimal residual disease, and early log reductions in *BCR-ABL* transcript levels, especially a 1-log reduction at three months, predicted MMR (major molecular response) at the 12-month milestone. In the referenced study between the evaluable patients at the 12 to 24 months milestone, of which % achieved MMR, where 24.5% achieved complete molecular response (CMR), and 44.9% achieved partial molecular response (>0.2 and <3-log reduction). This resulted in a cumulative response rate for molecular response of 69.4%, a notable fraction. We observed 35% of our study cases experiencing TKI failure with Imatinib which is concordant with reports by Senapati J et al. (2023) (40%) and Bhamidipati PK et al.(2013) (33%)[58][59].

In our study, 13% of patients who were initially on Imatinib shifted to 2nd generation TKIs due to suboptimal response, this was concordant with findings of Senapati J et al. (2023) (26.5%)[58]. Out of the 12 CML patients who constituted the TNR group in our study, IRMA testing was available for only 3 patients (25%) and out of them one patient (33%) harboured resistant *ABL1* TKD mutation which helped adjust the therapeutic plan for the patient. Although, qPCR is a sensitive and reliable technique to detect treatment failure among CML patients, further investigations are warranted to conclusively determine the cause for TKI failure, based on which definitive treatment decisions can be made. However, due to financial limitations among our population, majority of the patients are unable to afford these subsequent tests. In such resource constrained settings, qPCR assumes an even more important role in guiding the treatment of CML patients and monitor the response after any intervention.

Among the TR group we observed 75% patients had achieved deep molecular response which is also similar to the data reported by the previous study. (82%) Multiple studies have reported a higher incidence of DMR among Indian CML patients. The reason for this observation requires further investigation. Achieving and maintaining DMR is an essential criteria for patients to be eligible for treatment free remission and qPCR helps to select patients who would qualify for TFR.

It is important to highlight that while the patients who achieved CMR status reached undetectable *BCR-ABL* transcripts, which would suggest disease eradication, some patients still experienced molecular relapse after discontinuous therapy.

CONCLUSION

This study offers a thorough look at the role of qPCR and other commonly available laboratory techniques which find application in prognostication and therapeutic monitoring among CML patients. Our results confirm that CML mainly affects men and tends to affect patients at a younger average age in developing countries, which aligns with wider demographic patterns. We analysed the current international diagnostic and therapeutic guidelines in CML and integrated them in our study process. There's a notable inverse relationship between how well patients respond to treatment and certain molecular markers (%NCN and %IS-NCN), highlighting the need for effective therapies and careful molecular monitoring. Haematological factors, especially the white blood cell (WBC) count, proved to be solid indicators of how the disease progresses and how patients respond to treatment. Importantly, this study points out the effectiveness and cost-effectiveness of Indian generic imatinib mesylate (IM), making it a strong option for first-line treatment in settings with limited resources. The use of RQ-PCR for early detection of molecular response and tracking minimal residual disease further enhances its role in tailored disease management. However, the occurrence of molecular relapse in patients who initially achieved a complete molecular response highlights the importance of ongoing monitoring after treatment. This study also emphasises the need for reproducible and standardised MRD protocols to better manage these patients. Overall, this study provides valuable insights into the role of molecular diagnostic in managing CML and emphasizes the need for continuous assessment of both clinical and molecular factors to improve patient outcome.

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ABSTRACT

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm associated with the Philadelphia chromosome, which is a result of the t (9;22) (q34; q11) translocation and the BCR::ABL1 fusion gene. The BCR::ABL1 fusion gene encodes a constitutively active tyrosine kinase that leads to unregulated cell proliferation. Tyrosine Kinase Inhibitors (TKIs) such as imatinib have changed CML from a fatal heath issue to a chronic, manageable disease. However, the importance of minimal residual disease (MRD) remains important to demonstrate effectiveness of treatment, modify therapies, and evaluate treatment-free remission (TFR). In this study, quantitative real-time PCR (qRT-PCR) was performed to identify BCR::ABL1 transcript levels in peripheral blood samples from patients with CML treated with TKIs. Molecular response was evaluated by calculating the normalized copy number (NCN) and values on the international scale (IS).Molecular data were compared to routine hematological tests. The qRT-PCR assay was sensitive and could detect deep molecular responses such as MR4, MR4.5, and MR5 in some patients. ANOVA demonstrated statistically significant differences were demonstrated for molecular response, based on the different hematological baselines of each patient. This study confirms the superiority of molecular methods compared to conventional methods, using conventional response measures to monitor response to treatment and the disease. There have been improvements, but barriers remain in India, including the cost, access, and standardization of MRD testing. As MRD assessment is critical for personalized CML management and improving long-term outcomes, it is of utmost importance to incorporate MRD evaluation within the standard treatment pathway. Future opportunities may include using digital PCR and what machine learning algorithms might do to evaluate MRD quantification and predictive power.

Keywords:
Chronic Myeloid Leukemia, BCR::ABL1, Tyrosine Kinase Inhibitor, Minimal Residual Disease, Real-time PCR, Molecular Monitoring

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Introduction

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder in which the Philadelphia (Ph) chromosome is generated through translocation, t(9;22)(q34;q11) leading to a BCR-ABL1 fusion gene. This alteration leads to constitutive tyrosine kinase signaling and myeloid cells that cannot control their proliferation.Even so, even with deep molecular responses, residual leukemia may remain, and accurate assessment of Minimal Residual Disease (MRD) can be difficult without sensitive monitoring techniques.

The identification and quantification of MRD has significant implications clinically, as even small quantities of residual disease may lead to relapse. MRD assessment is important to understanding the depth of response, tailoring treatment, and when deciding whether to continue or discontinue therapy altogether[2]. In chronic myeloid leukemia (CML), monitoring MRD is especially salient to evaluating the effect of TKIs, understanding the risk of disease progression, and identifying patients who are candidates for treatment discontinuation based on strict molecular response criteria[1]. MRD monitoring on an ongoing basis has also highlighted the importance of delayed relapse detection, which offers an opportunity for timely therapy and overall better outcomes.

Because of its sensitivity and specificity, qPCR for BCR-ABL1 transcripts is the gold standard for MRD detection. New technologies, including digital PCR (dPCR) and next-generation sequencing (NGS), are improving the precision of next-generation MRD detection, with even fewer disease burden qualitative changes and an improved reproducibility of reporting. Flow cytometry and other molecular diagnostics also provide complimentary monitoring data for disease monitoring, providing mechanisms for MRD detection. Artificial intelligence and machine learning are increasingly explored for MRD reporting as well to improve data interpretation and predictive accuracy[4].

Even though MRD assessment is being advancing globally, there are still challenges concerning standardization and broader uptake of these techniques. In India, MRD monitoring techniques continue to be an area of focus in both clinical and research applications. The routine use of MRD assessment in the management of CML can be impacted in factors such as cost, access to molecular tests, and variability in testing protocols[3]. Additionally, the lack of consistency in MRD guidelines for commands across laboratories can contribute to variability in patient management. Addressing these challenges through standardized protocols, improved uptake of molecular testing, and incorporating MRD into routine clinical practice will assist with developing personalized treatment modifications and ultimately lead to improved outcomes. This review seeks to review the latest advances in MRD detection methods in CML, with a significant focus on their clinical relevance in the context of new technology and implementation challenges in Indian healthcare settings[5]. By discussing the existing and evolving MRD monitoring methods, this review should provide an overview of MRD monitoring in relation to CML management, its implications for patient care, and its future potential. It will also

discuss the importance of MRD-guided treatment decisions in relation to treatment-free remission (TFR) and long-term disease control.

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Materials and Methods Our study was conducted in the molecular pathology laboratory at Netaji Subhash Chandra Bose Cancer Hospital, Kolkata, West Bengal. Consecutive CML patients whose diagnosis was confirmed by hematological and molecular assays, were included in our study over a period of three months from January till March 2025. Peripheral blood sample which was collected from these patients were simultaneously analyzed in the hematology lab and molecular pathology lab. The sample collected was first analyzed using automated hematology cell counters (5 part, Sysmex). Based on the total WBC count, appropriate amount of sample was processed for molecular testing which involved RNA extraction from WBCs, RNA quantification and quality check and quantitative BCR::ABL1 analysis utilizing real time PCR technique. Sample collection: A total of 8-10cc peripheral blood was collected and equally divided in 3 EDTA vacutainers. One sample was sent for hematological analysis while the rest were processed for molecular testing. RNA isolation: Total RNA was extracted from blood sample using the NucleoSpin® RNA Blood Kit. This kit processes by direct total blood lysis from 200 – 400 µL whole blood collected in standard (e.g., EDTA) blood collection tubes. One of the most important aspects in RNA purification is to prevent RNA degradation during the isolation. With the NucleoSpin® RNA Blood method, leukocytes (the main source of RNA in whole blood) and other blood cells, are lysed by incubating the whole blood in a solution containing large amounts of chaotropic ions. A complex selective erythrocyte lysis and preparation of a leukocyte pellet is not necessary. Contaminating DNA, which is also bound to the silica membrane, is removed by a recombinant DNase solution which is directly applied onto the silica membrane during the preparation. Simple washing steps with two different buffers remove salts, metabolites, and macromolecular cellular components. • Add 350µL MDB (Membrane Desalting Buffer) onto the column and centrifuge 30s at 11000 x g. 5. Digest DNA • Add 95µL rDNase onto the column. Incubate at room temperature for 15 min. 6. Wash and dry silica membrane 1st wash • Add 200µL Buffer RB2 to the NucleoSpin® RNA Blood Column. Centrifuge for 30s at 11,000 x g. Discard flowthrough and Collection Tube and place the column into a new Collection Tube (2mL; provided) 2nd wash • Add 600µL Buffer RB3 to the NucleoSpin® RNA Blood Column. Centrifuge for 30s at 11,000 x g. Discard flowthrough and place the column into a new Collection Tube (2mL; provided). 3rd wash • Add 250µL Buffer RB3 to the NucleoSpin® RNA Blood Column. Centrifuge for 2min at 11,000 x g. In this step, ethanol is removed from the column. Place the column into a nuclease-free Collection Tube (1.5mL, supplied) and discard the Collection tube with flowthrough from the previous step. [If for any reason the liquid level in the Collection Tube has reached the NucleoSpin® RNA Blood Column after centrifugation, discard flow-through, and centrifuge again.] 7. Elute RNA • Add 60µL RNase-free H2O (supplied) onto the column and centrifuge 30s at 11,000 x g. The RNA is eluted into the Collection Tube. STATISTICAL ANALYSIS Statistical analysis of experimental data was done to see whether the differences among groups were

significant. One-way analysis of variance (ANOVA) was done manually for each dataset, providing a means of assessing whether the means of each group differed significantly from one another. For the manual analysis, we calculated the total sum of squares (SST), sum of squares between groups (SSB), and sum of squares within groups (SSW), and calculated the mean squares (MSB and MSW). The F-values were scored as the mean square between groups over the mean square within groups (F=MSB/MSW). They were then converted to an estimated p-value using the online stat calculator from GraphPad (https://www.graphpad.com/quickcalcs/pvalue1/), which took the F-ratios and converted them to p-values based on the degrees of freedom established by each dataset, ensuring accurate statistical representation. The F-ratio collected

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Digest DNA • Add 95µL rDNase onto the column.
https://www.takarabio.com/documents/User%2520Manual/NucleoSpin%2520TriPrep%2520DNA/NucleoSpin%2520TriPrep%2520DNA-RNA-Protein%2520Purification%2520User%2520Manual_Rev_05.pdf%3Fsrsltid%3DAfmBOoonkWsIRJ_YOVqyvoF55kmSVxblsvV--2PI9ukUz3GLD7KdFfGe

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Review of Literature
1.Chronic Myeloid Leukemia
It can arise at any age. With the emergence of research and use of the term Chronic Myeloid Leukemia (CML) in the early 1900s, scientists and clinicians were distinguishing CML from acute leukemias, observing that CML was a slower developing disease that primarily afflicted adults, while aggressive acute leukemia was more prevalent in children.
The essential genetic defect (related to CML) is an alteration recognized as the Philadelphia chromosome, which is the functional fusion of the BCR gene on chromosome 22 and ABL1 gene on chromosome 9.[5]. When it is not treated, the symptoms of CML worsen over time as the cancer proliferates.
In 1998, the first tyrosine kinase inhibitor (TKI), Imatinib (Gleevec), which specifically targeted the BCR::ABL protein and inhibits its activity, was developed. Imatinib was a game changer for CML, changing it from a fatal disease to a chronic condition that could be managed for a number of patients[6].
Now most patients with CML are living as if they have a manageable chronic disease. Many patients are expected to live normal lifespans with routine monitoring and treatment. The clinical goal for monitoring CML is to have patients in molecular remission, where the BCR::ABL gene is no longer detectable in the blood. Molecular monitoring refers to the monitoring that intercepts the amount of BCR-ABL fusion gene and activity. The two most common modalities for monitoring therapy are quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR).
1. a. CML in World & India:
It is most often diagnosed in adults, especially those aged between 45 and 55. The age at diagnosis is usually around 50 to 60 years. Childhood occurrences are quite rare, accounting for around 2-3% of all CML cases. he disease comes into being in three stages: chronic phase, with asymptomatic symptoms, accelerated phase, characterized by the deterioration of blood counts, and blast crisis, which is the acute and aggressive phase (akin to acute leukemia). The pathophysiologic basis of CML is genetic mutations causing unlimited growth of myeloid cells (a white blood cell) and abnormal blood cell formation.
The most characteristic feature of CML is the Philadelphia chromosome, a distinct genetic anomaly in leukemia cells' DNA. It occurs as a result of a translocation between chromosomes 9 and 22, designated as t(9;22)[8]. in normal circumstances, tyrosine kinases take part in signaling pathways that control cell growth, survival, and differentiation. The aberrant signaling produces both autocrine (stimulating self) and paracrine (stimulating nearby cells) effects on cell growth and survival, perpetuating the proliferation cycle[9]. Tyrosine kinase inhibitors (TKIs) revolutionized the treatment algorithm of chronic myeloid leukemia (CML) in the past two decades, with the most well-known being imatinib (Gleevec).

Prior to TKIs, the median survival time for CML patients was merely 3–5 years, with treatment modalities limited to chemotherapy and bone marrow transplantation. TKIs have made CML an intelligently chronic illness, with a 10-year survival rate of>80–90% in developed countries[10]. First-line TKIs, such as imatinib, dasatinib, and nilotinib, are presently available to block this BCR-ABL1 fusion protein and manage the disease process. Long-term, the original mutation responsible for the BCR-ABL fusion gene may result in additional genetic mutations and clonal evolution of leukemia cells. These secondary mutations may lead to more aggressiveness of the disease and drug resistance (particularly tyrosine kinase inhibitors such as imatinib).

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For these patients who are resistant or intolerant to first-line TKIs, new-generation drugs (such as bosutinib, ponatinib) could be used. Advanced disease patients don't gain any advantage from TKIs, therefore allogeneic stem cell transplantation is utilized [11]. In India, CML is a different scenario compared to the Western world, where it accounts for 15% to 20% of all types of leukemia. Among Indians, the rate of CML in leukemia has been estimated as high as 30% and 60%. Imatinib was originally highly costly and imposed heavy economic costs on patients until the advent of generics. Despite this, second- and third-generation TKIs like dasatinib and nilotinib are still highly costly and inaccessible to a vast majority of patients. Potentially curative bone marrow transplantation can be performed only in specialized centers and remains economically beyond the means of most patients in India [13]. 1.b. CML Pathogenesis Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm defined by the presence of the Philadelphia chromosome, which originates from a reciprocal t(9;22)(q34;q11) translocation. The t(9;22) produces the BCR::ABL1 fusion gene encoding a tyrosine kinase that is constitutively active, leading to the proliferation of myeloid cells without control. CML's tyrosine kinase homogeneously activates several downstream signaling pathways to support cell proliferation and suppress apoptosis [14]. The BCR::ABL1 chimeric protein activates numerous signaling cascades resulting in aberrant signal transduction pathways which ultimately lead to leukemogenesis. Furthermore, BCR::ABL1 inhibits mechanisms of DNA damage response causing genomic instability and also interferes with the integrin and chemokine signaling thereby facilitating leukemic cell escape from immune detection [15]. CML is also marked by progression from chronic phase (with comparatively normal differentiation) to accelerated and ultimately blast crisis phase (transformation into acute leukemia) which mainly occurs due to acquisition of additional mutations and mounting genetic abnormalities [16]. Philadelphia Chromosome: The Philadelphia chromosome is a short chromosome 22 due to a reciprocal translocation between chromosomes 9 and 22, defined as t (9; 22) (q34; q11). This is a characteristic genetic abnormality of chronic myeloid leukemia and has been observed in certain instances of acute lymphoblastic leukemia and acute myeloid leukemia [17] [18]. The chromosome was identified as early as 1960, when Nowell and Hungerford originally reported a small abnormal chromosome in CML patients [17]. The translocation forms the BCR::ABL1 fusion gene, the product of which encodes a constitutively active tyrosine kinase with a central role in leukemogenesis because it can induce self-sustaining cell growth, suppress apoptosis, and modify cell adhesion [19]. The BCR::ABL1 fusion gene results from juxtaposing the BCR gene from chromosome 22 and the ABL1 gene from chromosome 9[20]. The p210 isoform is most commonly associated with CML, whereas p190 is predominantly found in Ph-positive ALL. The p230 variant, although rare, is linked with a more indolent form of the disease. BCR::ABL1 exhibits persistently high tyrosine kinase activity, leading to the activation of multiple signaling pathways, including JAK-STAT, PI3K-AKT-mTOR, and RAS-MAPK. These pathways promote malignant transformation by enhancing

proliferation, survival, and genomic instability [18]. Furthermore, BCR::ABL1 alters the bone marrow microenvironment, contributing to leukemia stem cell maintenance and disease progression. CML goes through three different phases. The chronic phase is characterized by the accumulation of mature granulocytes while symptoms are minimal. The accelerated phase is characterized by increased blasts, worsening cytogenetic abnormalities, and therapy resistance. The blast crisis, resembling acute leukemia, presents a predominance of either myeloid or lymphoid blasts [21]. Additional mutations such as IKZF1, CDKN2A/B deletions, and TP53 mutations often support the progression of the disease and resistance to therapy [19]. While Ph is usually associated with CML, it is also found in 11-29% of adult ALL cases, as well as in a smaller fraction of AML cases. In Ph-positive ALL, the p190 isoform of BCR::ABL1 predominates and leads to a more aggressive course of the disease with a poorer prognosis as compared to Ph-negative ALL. In AML, Ph is rarely detected; it could suggest some unique biological status that would demand specific attention in therapy [17]. The introduction of TKIs, such as imatinib, represents a paradigm shift in the therapy of Ph-positive leukemias. Imatinib binds to the ATP-binding site of BCR-ABL1, inhibiting the kinase activity of this oncoprotein and, in turn, upstream kinase signaling [18]. Second- and third-generation TKIs such as dasatinib, nilotinib, bosutinib, and ponatinib have been made to counter the effect of resistance mutations as T315I. However, leukemia stem cells can persist in the presence of TKIs [21]. It is thus deemed of utmost necessity to develop additional treatment strategies such as combination therapies, allogeneic HSCT, and new agents targeting alternative signaling pathways to eradicate them [19]. It is among the most well-defined genetic alterations in hematologic neoplasia. Although TKIs have dramatically enhanced outcomes for Ph-positive leukemia patients, challenges remain in the form of drug resistance and minimal residual disease. Research into targeted therapies and combination approaches is still ongoing, raising hopes for better treatment and, possibly, cures [19]. 1. c. Phases of CML: Chronic myeloid leukemia (CML) is defined by three phases in disease progression: chronic phase, accelerated phase, and blast crisis phase. The classification of these phases is based on the proportion of blast cells present in the peripheral blood and bone marrow, as well as the associated clinical symptoms. The progression from one state to another are associated with a gradually worsening condition along with the increased improbability that

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Chronic Phase: It is the earliest and most prolonged stage of CML, accounting for an estimated 85 to 90% of cases at diagnosis [23]. It is characterized by the slow accumulation of mature and immature myeloid cells in the bone marrow and peripheral blood. In this phase, the percentage of blast cells in the peripheral blood or bone marrow stays below 10%. Clinically, the majority of cases present either with mild and nonspecific symptoms, such as fatigue, weight loss, or night sweats, and splenomegaly, or they're asymptomatic. This phase shows good response to TKIs, such as imatinib, dasatinib, and nilotinib. With good treatment and periodic monitoring, patients in this phase can control the disease indefinitely and enjoy a near normal life expectancy[24].
Accelerated Phase: This phase results from disease progression and is characterized by the increased proliferation of immature myeloid cells, indicating first-line treatment failure. The accelerated phase is defined by an increase in blast cells to between 10% and 19% in the peripheral blood or bone marrow. The accelerated phase is marked by a blast cell percentage ranging from 10% to 19% in the blood or bone marrow. The signs and symptoms are more pronounced; for example, persistent fever, night sweats, unintentional weight loss, worsening anemia, and splenomegaly. Devastatingly, some patients may show an increasing white blood cell count even on treatment. Here, first-generation TKIs are less effective, thus requiring second- or third-generation TKIs like bosutinib and ponatinib. A diagnosis is confirmed when blast cells make up 20% or more of the total cells in the blood or bone marrow \[16].

Blast Phase: This is the most advanced and aggressive phase of CML, with a prognosis and outlook comparable to that of acute leukemia. An uncontrolled proliferation of blast cells leads to severe marrow failure and systemic complications in these patients. It is diagnosed when blast cells account for 20% or more of the blood or bone marrow cell population [16].

Patients in this phase have severe infections, episodes of recurrent bleeding, extreme fatigue, bone pain, and organ infiltration, owing to the dramatically high blast cell counts. Life-threatening complications are considerably more likely. This phase has poor responses to TKIs, and management requires intensive chemotherapy, targeted therapies, and consideration for allogeneic HSCT. The prognosis is severely dismal, with most patients living only for a few months without aggressive intervention[25].

Based on the classification of CML into three phases, prognosis and treatment decisions can be determined. Survival outcomes following the chronic phase are much better with an early diagnosis and introduction of TKI therapy. Regular molecular monitoring of BCR-ABL1 transcript levels plays a crucial role in monitoring disease progression and detecting resistance mutations[24]. Upon progressing to the accelerated or blast phases of the disease, these individuals require early interventions to ensure a better therapeutic outcome and to stymie the further deterioration of their condition.

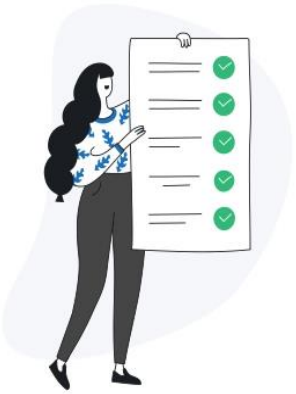
1. d. Clinical Features:

Many patients may be asymptomatic at the time of diagnosis, with CML becoming a chance finding following routine blood tests. These blood tests show generally an increased white blood cell count (leukocytosis), often with a predominance of myeloid cells, and occasionally thrombocytosis, that is, an increased platelet count. However, with disease progression, a spectrum of symptoms starts to develop in patients[26].

Common symptoms of CML include persistent fatigue and weakness, which result from anemia and the increased metabolic demands of the excessive number of abnormal cells. Another common feature is splenomegaly or an enlarged spleen, causing abdominal discomfort or pain; thus, patients may also complain of a sensation of fullness or early satiety due to an enlarged spleen[27]. Night sweats and occasional low-grade fever may occur because of the disease itself or secondary infections. Unintentional weight loss may also occur, stemming from increased energy expenditure and metabolic changes due to the disease. Another symptom experienced by some patients is bone pain attributed to some proliferation of myeloid cells in the marrow[28].

Though there are elevated white blood cell counts, CML patients may have a higher vulnerability to infections as a result of their impaired functionality. Other complications due to thrombocytosis include thrombosis (clots) or bleeding due to dysfunctional platelets. As CML develops, it can also enter the second phase of accelerated CML with abundant immature cells, or blasts in the blood or the bone marrow. The disease finally goes into a blast crisis in which features of acute leukemia manifests, due to increase in blast cells (over 20% in the blood or bone marrow). Symptoms during this phase include intense fatigue, fever, and increased bleeding or bruising[26].

Complete blood counts in CML usually show leukocytosis, with a left shift; with increased numbers of immature myeloid cells. There could be anemia and even thrombocytosis. Bone marrow biopsy in these patients may show hyper cellularity with increased numbers of myeloid cells. Confirmation of diagnosis requires cytogenetic analysis that reveals the Philadelphia chromosome or the BCR-ABL fusion gene protein[4].



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A complete blood count (CBC) is the initial and one of the most important diagnostic tests in suspected cases of CML. The CBC findings often reveal moderate leukocytosis in excess of 100,000/mm³. A peripheral blood smear shows a differential count on the left shifted side: that is, an increase in immature myeloid precursors such as myelocytes, metamyelocytes, and promyelocytes within the granulocytic lineage. These features of peripheral blood smear are often referred to as the "myelocyte bulge." This is associated with a leukocytosis, with white blood cell counts ranging from about 50,000/mm³ to >300,000/mm³. CML is frequently associated with the presence of basophilia and eosinophilia, which allow differentiation from reactive leukocytosis. Usually, there is also normocytic or normochromic anemia because of bone marrow suppression and ineffective erythropoiesis[29].

Thrombocytosis elevates the platelet counts and is observed in some patients with early cases of the disease, while increasingly severe cases result in thrombocytopenia. The range of increase in platelets can vary for different patients, and in some cases, count levels can go above 1,000,000/mm³. Important findings in the CBC of CML patients are absolute neutrophilia, usually with hyper segmented neutrophils[30].

While leukocytosis can be seen in other conditions, the characteristic left shift in the granulocytic lineage along with basophilia, sets it apart from other conditions. In In CML, the leukocyte alkaline phosphatase (LAP) score is typically low, whereas it is characteristically elevated in a leukemoid reaction.

Follow-up of CBC parameters is also important in assessing the response to treatment, since with adequate treatment there will be a gradual normalization in all hedion levels[29].

Moreover, further information relating to the disease progress can be gathered from a close morphological study of the peripheral blood smear. An increase of circulating blast cells might indicate that the disease has transformed into accelerated or blast phase. Regular monitoring of CBC will not only assist in deciding the therapy to be undertaken on a case-to-case basis but will also help spot early signs of resistance or possible relapse of the disease. Persistent eosinophilia and basophilia in the treated disease could allude to an inadequate response, deserving further molecular and cytogenetic analyses[30].

2. b. Bone Marrow:

A bone marrow aspiration and biopsy can be of significant help in making known the mechanism of the disease and staging CML. The marrow in CML during the chronic phase is usually hypercellular and the myeloid-to-erythroid (M: E) ratio is often greater than 10:1. There is a decrease in the number of erythroid precursors, while megakaryocytes may show abnormal clustering and hyper lobulation. Granulocytic hyperplasia is another prominent

feature with a background of increased myeloid precursors at various stages of differentiation. Typical symptoms of CML include persistent fatigue and weakness, primarily caused by anemia and the

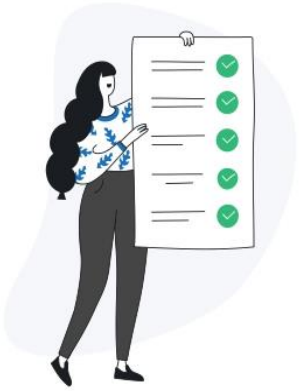
elevated metabolic demands of the excessively produced leukemic cells [31].

In certain cases, reticulin staining may reveal increased fibrosis within the bone marrow. Marrow fibrosis has been associated with disease progression and poor prognosis. Bone marrow analysis is a valuable tool in distinguishing between CML and other myeloproliferative neoplasms such as polycythemia vera or primary myelofibrosis. Cytogenetic analysis of bone marrow cells will detect additional chromosomal abnormalities, known as clonal evolution, which may signal disease progression. Certain anomalies have been related to transformation into accelerated or blast phase such as trisomy 8, isochromosome 17q, and extra Ph chromosomes[32].

A bone marrow examination is mandatory if the molecular and cytogenetic studies are inconclusive such as in case of atypical CML or additional mutations, in such patients, it can reveal the specific cross-sectional histology pattern of disease progression. It additionally provides information on the level of hematopoietic suppression, which is important to decide on therapies mainly in the context of intensive treatment or stem cell transplantation[31].

2. c. Lactate Dehydrogenase (LDH):

Lactate dehydrogenase is an enzyme that serves as an indirect marker of tissue turnover and cell destruction. In CML, elevated LDH levels correlate with increased metabolic activity and disease burden. Higher LDH levels may indicate disease progression to the accelerated or blast phase and help in prognostication. Elevated LDH at diagnosis suggests a high leukemic cell turnover. A reduction in LDH levels following the initiation of TKI therapy indicates a favorable therapeutic response. Persistently high or rising LDH levels may reflect disease progression or resistance to therapy[33]. Beyond its diagnostic value, LDH levels are increasingly being explored as a potential marker for treatment response stratification. Patients with persistently high LDH levels despite achieving hematological remission may require closer molecular monitoring. Elevated LDH is also associated with extramedullary disease manifestations, such as hepatosplenomegaly, which is commonly seen in advanced CML[33].



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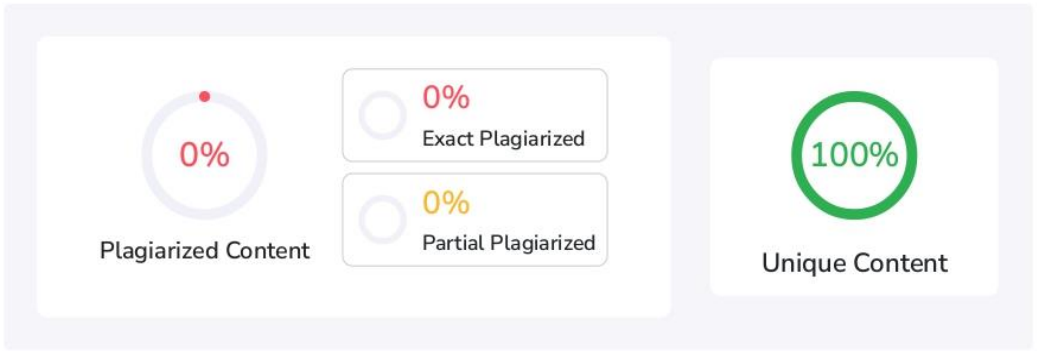

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Karyotyping: Karyotyping is an essential diagnostic and monitoring tool in CML which is used to detect numerical and structural chromosomal abnormalities. This technique is primarily used to detect the Philadelphia chromosome and additional chromosomal abnormalities. It helps in diagnosing the disease, predicting prognosis, and assessing response to therapy. Karyotyping is performed on bone marrow metaphase cells using Giemsa (G-banding) staining. During disease progression, clonal evolution may occur, leading to additional chromosomal abnormalities (ACAs) which are detectable by this technique. Some common ACAs include: Trisomy 8, Isochromosome 17q, Additional Philadelphia chromosome, etc[34]. 2. e. Fluorescence In-Situ Hybridization (FISH) for the Philadelphia Chromosome: The FISH technique is used in cytogenetics to detect the Philadelphia (Ph) chromosome, which is specific to CML. FISH has many advantages over conventional karyotyping in diagnosing CML. Utilizing FISH, BCR::ABL1 fusion gene can, therefore, be viewed in interphase nuclei with rapidity and sensitivity [35]. FISH is of particular value in cases where standard cytogenetics fail due to poor metaphase yields or true cryptic translocations that are not visible on a standard karyotype. It detects minimal residual disease through the detection of low levels of Ph-positive cells following treatment, providing an early clue regarding disease relapse. This technique can be applied to peripheral blood samples, bone marrow aspirates, or even fixed tissue specimens, thus allowing great flexibility in testing[36]. The latest improvements in FISH technology have resulted in FISH and SKY, which greatly improve the ability to detect complex chromosomal aberrations associated with the progression of CML. These methods are a major advance for detecting further cytogenetic aberrations such as secondary translocations, deletions, or duplications, which may confer resistance to the tyrosine kinase inhibitors[36]. FISH is essential for monitoring treatment response. FISH follow-up shows a lowering percentage of Ph-positive cells, hypothesizing good therapeutic response; otherwise, when Ph-positive cells are persistent under treatments, which suggests inadequate response or an emergence of resistance, thus requiring further molecular investigations[33]. 2. f. Overview of Molecular Diagnostics in CML: Molecular diagnostics justify their relevance in the diagnosis, monitoring, and assessment of CML treatment response. Polymerase chain reaction (PCR) is the primary molecular technique used to detect BCR::ABL1 transcripts. Reverse transcriptase PCR (RT-PCR) is utilized for the qualitative detection of the BCR::ABL1 fusion transcripts, and can detect them either in blood or bone marrow samples. RT-PCR has a very high sensitivity thus necessitating its use as a key technique for monitoring MRD[32]. For monitoring treatment response in CML, quantitative Real time PCR (qPCR) is considered the gold standard due to the possibility of very precise quantification of BCR::ABL1 transcript levels. A major molecular response (MMR) is defined as 3-log reductions of BCR::ABL1 transcripts from the baseline, while deep molecular response (DMR) is defined as undetectability or extremely low transcript levels below 4.5 log reductions of BCR::ABL1 transcripts from the baseline. Regular qPCR monitoring gives an insight into therapeutic efficacy by detecting early signs of relapse.

NGS is another molecular technique which is increasingly being used to detect additional mutations responsible for resistance towards TKIs, thus helping to guide personalized treatment strategies[37]. With the development of these high-throughput sequencing techniques, the novel genetic alterations associated with CML progression have been characterized, which enables the refinement of risk stratification models. In addition, digital droplet PCR has emerged as a highly sensitive technique to detect low-level mutations and MRD while enhancing the ability to track disease evolution[32]. Mutation analysis of the ABL1 kinase domain is critical for CML patients in whom first and second-generation TKIs became ineffective. Mutations like T315I are associated with resistance to 1st and 2nd generation TKIs, and options like ponatinib or allogeneic stem cell transplant would be considered. Only with comprehensive molecular profiling can more informed decisions be made regarding therapies that would optimize patient outcomes[37]. 3. Utility of Commonly Utilized Molecular Diagnostic tests in CML: 3 (a): Qualitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) for BCR::ABL1 testing Molecular tests play a key role in diagnosing and managing CML. To establish the diagnosis of CML, qualitative reverse transcription PCR is very useful. RT-PCR measures BCR::ABL1 mRNA levels compared to control genes like ABL1, GUSB, or BCR, which helps check sample quality. Unlike quantitative real time PCR (qRT-PCR), which measures transcript levels over time, qualitative RT-PCR confirms the presence or absence of the fusion gene and helps to determine the BCR::ABL1 transcript to be measured during follow up. Various BCR::ABL1 fusion transcripts result from differences in the breakpoint within the BCR gene, giving rise to distinct fusion protein isoforms: Table 9: Types of BCR::ABL1 Transcripts Detected by RT-PCR[38] Fusion Transcript Protein Product Frequency in CML e13a2 (b2a2) p210 BCR::ABL1 ~40-50% e14a2 (b3a2) p210 BCR::ABL1 ~50-60% e13a2 + e14a2 Both p210 variants ~10-15% e1a2 p190 BCR::ABL1 Rare in CML, common in Ph+ ALL e19a2 p230 BCR::ABL1 Rare, associated with neutrophilic CML (CML-N) 3 (b): Quantitative Real-Time PCR (qRT-PCR) for BCR::ABL1 testing Quantitative Reverse Transcription Real-time PCR (qRT-PCR) is the gold standard for monitoring molecular MRD in patients with CML. It quantifies BCR::ABL1 transcript levels over time, allowing precise assessment of treatment response and early detection of relapse. The principle of qRT-PCR involves three main aspects: 1.Reverse Transcription (RT): Converts BCR::ABL1 mRNA into complementary DNA (cDNA). 2.Quantitative PCR (qPCR): Uses fluorescent probes to amplify and quantify cDNA, measuring transcript levels in real-time. 3.Normalization: BCR::ABL1 transcript levels are compared to a control gene (e.g., ABL1, GUSB, B2M, BCR) to standardize results. Table 10: Summary of Key Equipment & Reagents required for qRT-PCR BCR::ABL1 quantitative testing [39] Equipment Type Example Models RNA Extraction System Extracts total RNA from peripheral blood or bone marrow. Thermal Cycler (RT-PCR) Converts extracted RNA into complementary DNA (cDNA) using reverse transcriptase enzyme. Real-Time PCR Machine Performs quantitative real-time PCR using fluorescent TaqMan probes or SYBR Green. qPCR Plates/Tubes 96-well or 384-well PCR plates or 0.2 mL PCR tubes (low-binding, RNAse-free). Pipettes & Tips RNAse-free, sterile filtered pipette tips (to prevent contamination). Fluorescent Probes Probes for BCR::ABL1 fusion detection. Analysis Software Analyzes BCR::ABL1 transcript levels relative to control genes (e.g., ABL1, GUSB). Reference Standards WHO

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qRT-PCR finds a wide range of application in CML including: baseline molecular assessment, treatment monitoring, early detection of resistance or relapse and even providing therapeutic markers guiding TKI discontinuation (Treatment-Free Remission, TFR).
Laboratories report qRT-PCR BCR::ABL1 results using the International Scale (IS) to make sure the results are reproducible universally. Doctors look at important molecular milestones to make treatment choices and see if patients can stop treatment while staying healthy (TFR). These milestones include early molecular response (EMR) major molecular response (MMR; ≤0.1% BCR::ABL1 IS), and deep molecular response (DMR; ≤0.01% BCR::ABL1 IS)[39].

3. b. Timing of Test as per international guidelines:

Molecular testing for chronic myeloid leukemia is carefully scheduled at different time points during diagnosis and treatment, as per guidelines from reputed organizations like the European Leukemia Net (ELN) and the European Treatment and Outcome Study (EUTOS). The timing and regularity of these tests are crucial for accurately diagnosing the disease, keeping track of how well treatment is working, spotting any resistance, and determining if a patient is eligible for treatment-free remission.

Table 11: Recommended Timing of Molecular Tests in Chronic Myeloid Leukemia (CML) as per Current Guidelines

Clinical Stage Recommended Molecular Test and Timing

At Diagnosis

Qualitative RT-PCR to detect BCR-ABL1 fusion gene and identify transcript variant (e13a2/e14a2 or atypical forms) [39]

First year of TKI therapy Quantitative RT-qPCR every 3 months to monitor molecular milestones: ≤10% IS at 3 months (EMR), ≤1% IS at 6 months, and ≤0.1% IS (MMR) by 12 months [40]

After achieving stable MMR/DMR Quantitative RT-qPCR every 6 months to ensure ongoing molecular response maintenance [40]

Before Treatment-Free Remission (TFR) Confirm deep molecular response (MR4 or deeper) sustained for ≥2 years before discontinuing TKI [39]

After TKI discontinuation (TFR monitoring) Quantitative RT-qPCR monthly for the first 6 months, then every 2-3 months up to 18 months; long-term follow-up every 3-6 months to detect molecular relapse [39]

In case of treatment failure or loss of MMR Immediate BCR-ABL1 kinase domain mutation analysis to detect TKI resistance mutations; use of Sanger sequencing or NGS based on sensitivity requirements

molecular assessments.

Once a patient starts on tyrosine kinase inhibitor therapy, regular quantitative molecular testing kicks in to check how well they're responding. The ELN guidelines suggest that during the first year of treatment, quantitative RT-qPCR should be done every three months. These tests are essential for tracking whether the patient is achieving key molecular targets. At the three-month mark, achieving an early molecular response with BCR::ABL1 transcript levels at or below 10% on the International Scale (IS) is a strong indicator of positive long-term outcomes. Monitoring continues at six months to see if BCR::ABL1 levels fall to 1% IS or lower, and by the twelve-month point, patients should ideally reach a major molecular response, which is defined as BCR::ABL1 levels at or below 0.1% IS[40]. If these milestones aren't met, it could indicate a less-than-optimal response or the development of resistance, which would require further assessment and possibly a change in treatment.

Once patients reach and maintain a significant molecular response, or even deeper levels like MR4 (BCR-ABL1 ≤0.01% IS) or MR4.5, they can cut back on how often they get tested. At this stage, the guidelines suggest doing molecular tests every six months. This less frequent testing is seen as enough to keep the disease under control while avoiding unnecessary procedures. However, if there is any unexpected rise in BCR-ABL1 transcript levels during this time, it's crucial to reassess the situation right away to check for any potential loss of response or disease progression[40].

For patients who manage to maintain a stable deep molecular response for at least two years, stopping TKI therapy to pursue treatment-free remission can be a viable option. However, it's important to note that the risk of molecular relapse is highest right after treatment is halted, so a more rigorous molecular monitoring schedule is crucial. According to the ELN guidelines, molecular testing should be done monthly for the first six months after discontinuing TKI therapy. After that, testing should continue every two to three months for at least the next twelve to eighteen months to quickly catch any signs of molecular relapse[39]. If there is a sustained loss of MMR or an increase in BCR::ABL1 levels, it is necessary to restart TKI therapy to prevent a hematological relapse. When molecular testing shows rising BCR::ABL1 transcript levels or a complete loss of the previously achieved response, testing for BCR::ABL1 kinase domain mutations becomes essential.

This testing helps identify point mutations in the kinase domain of the BCR::ABL1 gene, which could lead to resistance against certain TKIs. Guidelines suggest conducting mutation analysis at these critical junctures to help determine the best alternative therapies. While Sanger sequencing has been the traditional method for this, next-generation sequencing (NGS) is gaining popularity due to its superior sensitivity and ability to detect low-level mutations that could impact clinical decisions[39][41].

Sticking to these recommended testing intervals is crucial for catching any less-than-ideal responses early on, spotting resistance mutations, and making smart choices about therapy changes or safe discontinuation. Regular molecular testing, along with standardized reporting on the International Scale, has played a significant role in enhancing the survival rates and quality of life for CML patients during the TKI era[39][40][41].

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4. c. RNA Extraction Technique and Quality Control: Thorough RNA extraction and quality control form an essential part of a molecular diagnostic test and treatment monitoring for CML. Fiber those, the realization of gene expression quantification, as well as an optimized RNA extraction technique, represent a key step for BCR-ABL fusion transcript detection. RNA Extraction Techniques: Several RNA extraction techniques have been explored for CML studies, particularly for isolating RNA from blood or plasma samples. The two most commonly used approaches include phenol-chloroform extraction (e.g., TRIzol method) and column-based extraction kits. a. TRIzol-Based Extraction: The TRIzol-based RNA extraction method is widely used because it effectively isolates high-quality RNA[45]. The method relies on the phase separation using phenol and guanidine isothiocyanate to isolate RNA while discarding DNA and proteins. Possible drawbacks include insufficient removal of contaminating genomic DNA and the need for meticulous practices to prevent RNA degradation. b. Column-Based Extraction Kits: Several commercial RNA purification kits have been developed that promise a better yield and purity of the RNA recovered. For instance, GeneAll® RiboEx™ showed that minor protocol adjustments made it superior to TRIzol for extracting high-quality RNA from plasma samples. Compared to TRIzol, column extraction methods allow the process to be conducted more straightforwardly, thereby lowering the risk of contamination[46]. The underlying principle of this technique is the selective adsorption of RNA to silica in the presence of chaotropic salts. The lysis buffer used in this method contains guanidinium salts to lyse cells and inactivate RNases. In high salt conditions, RNA binds to silica. This is followed by washing steps remove contaminants, after which finally the extracted RNA is eluted in RNase-free water or buffer. c. Magnetic Bead-Based Extraction This technique is based on the process of nucleic acid binding to magnetic beads coated with silica or polymer. Magnetic beads capture RNA in presence of chaotropic salts or binding buffers. Magnetic beads are then exposed to magnetic field which holds the beads during wash steps. Finally, RNA is eluted in low-salt buffer or water. c. Direct Lysis Methods In this method the cells are lysed in a buffer that stabilizes RNA and inhibits RNases. This is a crude method where the lysate can be directly used in RT PCR reactions. However, this is a simplified approach for rapid analysis when RNA purity is less critical. Quality Control (QC) in RNA Extraction: The quality control of extracted RNA is vital because it guarantees that any of the downstream applications are reliable, like real-time quantitative polymerase chain reaction RT-qPCR. Typically, some common QC parameters will include the following. RNA yield and Purity: A spectrophotometer, such as a NanoDrop, can measure RNA concentration mainly by measuring absorbance values at 260/280 nm and 260/230 nm. A ratio close to 2.0 is generally considered a good indication of RNA purity. If the ratio is less than 2.0, it could mean that some proteins or phenol has contaminated the RNA preparation. A low 260/230 ratio typically indicates the presence of salt contamination [46]. RNA Integrity: RNA integrity is assessed using agarose gel electrophoresis or Bioanalyzer-based RNA Integrity Number (RIN) analysis. Highly intact

RNA should exhibit clear ribosomal RNA bands without significant degradation[45]. Reverse Transcription and qPCR Efficiency: The extracted RNA is subjected to complementary DNA (cDNA) synthesis by reverse transcription in support of real-time PCR analysis. The degree of amplification and the reproductive ability of housekeeping genes (GAPDH, ABL1) is taken as an indicator of RNA quality[43]. Therefore, samples with good RNA integrity produce qPCR results with consistency and reliability, important for minimal residual disease detection in CML patients. Optimization of RNA extraction techniques and the implementation of robust QC measures in CML diagnostics and treatment monitoring are essential. Both TRIzol-based and column-based methods offer distinct advantages. However, RNA purity and integrity always take precedence in any molecular analysis for reliable diagnosis and treatment. 4. d. Real-Time PCR Process: Quantitative PCR (qPCR) for Chronic Myeloid Leukemia is the foremost molecular technique to detect and quantify the BCR-ABL1 fusion gene due to the Philadelphia chromosome translocation (t9;22). The presence of a constitutively active tyrosine kinase promotes the uncontrolled proliferation of myeloid cells [47]. qPCR, therefore, finds itself very much in use for CML diagnosis, treatment monitoring, and minimal residual disease detection due to its respectably high sensitivity and specificity. The technique begins with obtaining peripheral blood or bone marrow samples from suspected or diagnosed patients with CML. Total RNA then is extracted using column-based methods or phenol-chloroform methods to get rid of contaminants and inhibitors that might interfere with the reaction [48]. Thereafter, the purity and concentration of RNA are assessed consecutively, for example, spectrophotometer or fluorometric quantification, to check whether the valuable input sample is suitable for use in downstream applications. As qPCR detects gene expression, not genomic DNA, extracted RNA is reverse-transcribed into cDNA using a reverse transcriptase enzyme. This reaction has primers directed against the BCR::ABL1 transcript and a reference gene, which is often ABL1, to act as an internal control for expression normalization with changes in sample input. The reverse transcription step ensures that all the RNA has been effectively converted into cDNA, which will then serve as a template for amplification in qPCR. A master mix is made up of the cDNA template, specific primers for BCR::ABL1 and ABL1, DNA polymerase, dNTPs, buffer, and a fluorescent dye or probe for real time detection. This procedure usually applies a TaqMan probe-based method for qPCR detection, characterized by high specificity. This approach uses a probe that is specific to a sequence and is labeled with a fluorophore and a quencher, which gives off fluorescence as the probe is degraded in a reaction thought to be progressing during amplification[48]. Alternatively, SYBR Green dye, which binds to double-stranded DNA, can be utilized; however, the amplification specificity has to be verified through melt curve analysis. The qPCR reaction is performed in a thermal cycler: initial denaturation of DNA at a high temperature to separate the strands, followed by cycles of denaturation, annealing of the primers initiate

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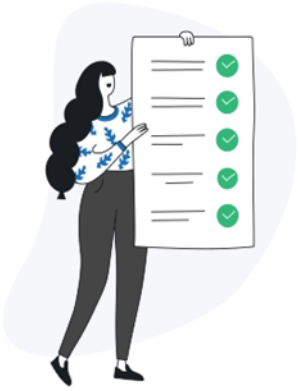
The quality controls offered during the process, from execution to completion, ensure the accuracy and reliability of the results. Non-template controls (NTCs) are incorporated to rule out possible contamination whereas positive controls assure the sensitivity of the assay [48]. After amplification, melt curve analysis is performed with SYBR Green to distinguish specific products from non-specific amplifications or primer-dimers. Triplicate assays are usually run in qPCR to ensure reproducibility, and the amplitude of detected signals assessed in qPCR can be used to compare variations in transcript levels in the samples tested. qPCR is one of the most crucial techniques in CML treatment monitoring, specifically for the patients taking TKI therapy such as imatinib, dasatinib, or nilotinib. With the assistance of continuous monitoring over time, qPCR allows the assessment of the molecular response by watching for the decrease of BCR-ABL1 transcript levels [47].
The identification of major molecular response (BCR::ABL1 ≤ 0.1% on the IS) and BCR::ABL1 transcript copy's decline during treatment demonstrates the success of therapy, whereas, on the other hand, resistant cases or relapses will come at some more definite levels, thus opening doors to the next level of intervention. qPCR being one of the most sensitive, rapid, and quantitative means assays not only enshrined itself as the gold standard for measuring molecular monitoring in CML but also guided important decisions such as identifying instances of treatment resistance.
4. e. Real Time PCR Analysis:
When examining qPCR results in Chronic Myeloid Leukemia, researchers look to spot and measure BCR::ABL1 fusion gene transcripts. These transcripts act as a crucial molecular sign for diagnosing keeping an eye on treatment, and finding tiny traces of the disease. The first thing analysts do is check the cycle threshold (Ct) value. This value shows the cycle where fluorescence goes above the background threshold[47]. A Ct value that's lower points to more BCR::ABL1 expression meaning the disease is more severe. On the flip side, a higher Ct value hints at fewer transcripts or possible recovery. In most cases, patients just diagnosed with CML have low Ct values because they have lots of leukemia cells. As treatment starts to work, patients' Ct values tend to go up as their BCR::ABL1 transcript levels go down.

RNA should exhibit clear ribosomal RNA bands without significant degradation[45]. Reverse Transcription and qPCR Efficiency: The extracted RNA is subjected to complementary DNA (cDNA) synthesis by reverse transcription in support of real-time PCR analysis. The degree of amplification and the reproductive ability of housekeeping genes (GAPDH, ABL1) is taken as an indicator of RNA quality[43]. Therefore, samples with good RNA integrity produce qPCR results with consistency and reliability, important for minimal residual disease detection in CML patients. Optimization of RNA extraction techniques and the implementation of robust QC measures in CML diagnostics and treatment monitoring are essential. Both TRIzol-based and column-based methods offer distinct advantages. However, RNA purity and integrity always take precedence in any molecular analysis for reliable diagnosis and treatment. 4. d. Real-Time PCR Process: Quantitative PCR (qPCR) for Chronic Myeloid Leukemia is the foremost molecular technique to detect and quantify the BCR-ABL1 fusion gene due to the Philadelphia chromosome translocation (t9;22). The presence of a constitutively active tyrosine kinase promotes the uncontrolled proliferation of myeloid cells [47]. qPCR, therefore, finds itself very much in use for CML diagnosis, treatment monitoring, and minimal residual disease detection due to its respectably high sensitivity and specificity. The technique begins with obtaining peripheral blood or bone marrow samples from suspected or diagnosed patients with CML. Total RNA then is extracted using column-based methods or phenol-chloroform methods to get rid of contaminants and inhibitors that might interfere with the reaction [48]. Thereafter, the purity and concentration of RNA are assessed consecutively, for example, spectrophotometer or fluorometric quantification, to check whether the valuable input sample is suitable for use in downstream applications. As qPCR detects gene expression, not genomic DNA, extracted RNA is reverse-transcribed into cDNA using a reverse transcriptase enzyme. This reaction has primers directed against the BCR::ABL1 transcript and a reference gene, which is often ABL1, to act as an internal control for expression normalization with changes in sample input. The reverse transcription step ensures that all the RNA has been effectively converted into cDNA, which will then serve as a template for amplification in qPCR. A master mix is made up of the cDNA template, specific primers for BCR::ABL1 and ABL1, DNA polymerase, dNTPs, buffer, and a fluorescent dye or probe for real time detection. This procedure usually applies a TaqMan probe-based method for qPCR detection, characterized by high specificity. This approach uses a probe that is specific to a sequence and is labeled with a fluorophore and a quencher, which gives off fluorescence as the probe is degraded in a reaction thought to be progressing during amplification[48]. Alternatively, SYBR Green dye, which binds to double-stranded DNA, can be utilized; however, the amplification specificity has to be verified through melt curve analysis. The qPCR reaction is performed in a thermal cycler: initial denaturation of DNA at a high temperature to separate the strands, followed by cycles of denaturation, annealing of the primers initiate



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In short, the suggested timeline for molecular tests in CML offers a well-organized and research-backed way to manage the disease effectively. Getting tested on time helps with accurate diagnoses, assessing the trend of therapeutic response, spotting any resistance early on, and keeping a close eye during attempts at treatment-free remission (TFR).

3. c. qRT-PCR vs Other Molecular Techniques in CML MRD

Managing chronic myeloid leukemia (CML) leans heavily on advanced molecular diagnostic techniques, especially RT-PCR and qPCR. While both methods are rooted in PCR technology and focus on amplifying nucleic acids, they have distinct goals, approaches, sensitivities, and roles in the clinical landscape of CML.

A key advantage of qPCR over RT-PCR is its capacity to provide standardized results on the International Scale (IS). This standardization was created to ensure consistency across laboratories, enabling reliable comparisons of BCR-ABL1 transcript levels worldwide[40]. The quantitative aspect of qPCR is essential for identifying specific molecular milestones, such as:

• Early Molecular Response (EMR): BCR-ABL1 ≤10% IS at 3 months

• Deep Molecular Response (DMR): BCR-ABL1 ≤0.01% IS or deeper

Hitting these milestones at specific times is linked to better long-term outcomes, like improved survival rates and the possibility of being eligible for Treatment-Free Remission (TFR)[39].

When it comes to sensitivity, qPCR really stands out compared to RT-PCR. While RT-PCR can effectively show whether BCR-ABL1 transcripts are present, qPCR takes it a step further by detecting transcript levels that are as low as a 4 to 5-log reduction from the standardized baseline. This heightened sensitivity is crucial for spotting minimal residual disease (MRD) and helps guide clinical decisions, such as whether to continue therapy, adjust doses, or even stop treatment in cases of sustained deep molecular response (DMR)[40].

That said, qPCR does have its drawbacks. Its accuracy hinges on thorough assay validation, the careful selection of internal control genes (usually ABL1 or GUSB), and the creation of lab-specific conversion factors (CFs) to align with the International Scale[40]. Plus, external quality assurance (EQA) programs are vital for ensuring consistency across different labs[39].

To sum it up, qualitative RT-PCR serves mainly as a diagnostic tool, crucial for confirming the presence and type of BCR-ABL1 fusion transcript at the initial diagnosis of CML. It gives a straightforward yes or no answer without detailing the disease burden. In contrast, qPCR is a quantitative, dynamic tool that's used throughout treatment to track disease progression, assess treatment response, and detect minimal residual disease with impressive sensitivity and accuracy. Both methods work hand in hand in managing CML, with RT-PCR laying the groundwork at diagnosis and qPCR steering therapeutic choices as the disease evolves.

Molecular Minimal Residual Disease (MRD) in Chronic Myeloid Leukemia (CML) refers to the detection

and quantification of BCR::ABL1 fusion transcripts using quantitative real-time PCR (qRT-PCR) after treatment with Tyrosine Kinase Inhibitors (TKIs). Molecular MRD monitoring helps assess treatment response, predict relapse, and guide treatment-free remission (TFR) strategies. Grasping the concept of MRD analysis is essential for effective management of CML.
The BCR-ABL1 fusion gene is the molecular signature of CML, and measuring it with advanced techniques is what MRD assessment is all about. Right now, the gold standard test for CML-MRD monitoring real-time quantitative reverse transcription PCR (RT-qPCR), thanks to its impressive sensitivity and specificity[41].

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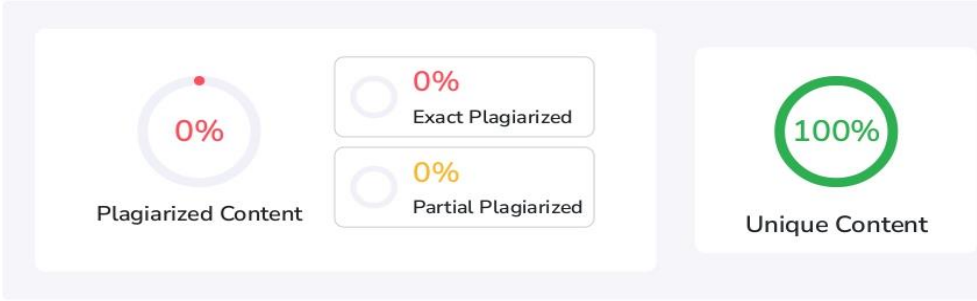

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The European LeukemiaNet (ELN) recommends that treatment response in CML be monitored using both conventional cytogenetics—specifically, chromosome banding analysis (CBA) of at least 20 metaphases from bone marrow cells—and real-time quantitative polymerase chain reaction (RT-qPCR). As previously stated, cytogenetic evaluations should be conducted at 3, 6, and 12 months until a complete cytogenetic response (CCyR) is achieved. After achieving a complete cytogenetic response (CCyR), chromosome banding analysis (CBA) can be substituted with fluorescence in situ hybridization (FISH) performed on a minimum of 200 nuclei from peripheral blood cells [49].

RT-qPCR should be performed every three months using buffy coat blood cells. ELN also states that when RT-qPCR methods are standardized and results are reported using the International Scale (IS) as BCR::ABL1%, this method alone may suffice for monitoring treatment response—not just after CCyR, but even from the outset—since RT-qPCR is more sensitive and does not require bone marrow sampling.

After achieving a major molecular response (MMR, defined as BCR::ABL1 ≤ 0.1% IS), RT-qPCR testing can be conducted every 3 to 6 months, depending on factors such as baseline risk, transcript levels, and their fluctuations. For patients who discontinue therapy following a stable, deep molecular response, monthly monitoring is advised for the first year, followed by testing at least every three months thereafter.

ELN categorizes responses into three clinical grades:

Optimal: The current therapy should be maintained, as the response suggests excellent outcomes and near-normal life expectancy.

Failure: A change in treatment is necessary, as the current response indicates a high risk of disease progression and mortality.

Warning: This intermediate category signals suboptimal response. Although better outcomes may be possible, there is insufficient evidence to recommend specific changes in therapy.

The response criteria for first-line imatinib treatment are supported by strong clinical evidence.
In contrast, response definitions for second-line treatments are provisional and primarily based on data from dasatinib and nilotinib.

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Results

Our study involved a total of 61 patients who were diagnosed with CML. The demographic and haematological characteristics of the enrolled CML patients are summarized in Table 2. In our study group, 65.5% of the patients were males and 34.5% were females. The median age in our study group was 46 years. We divided the patients in the study into younger age group with cases ≤ 46 years age (29.5%) and older age group with cases >46 years age (70.5%). (Table 2)

We divided our study cases into two groups: untreated patients (UT) 15% (n=9/61) and treated patients (TP): those patients who received treatment with tyrosine kinase inhibitors (TKI) 85% (n=52/61). The treated patients were further subdivided into two groups: Treatment responders (TR) and Treatment non responders (TNR) based on those patients who responded optimally to TKI (TR) and those whose response was considered “warning” or “failure” as per ELN-CML guidelines. 40 patients out of 52 were grouped as TR (77%) while 12 patients were grouped as TNR (23%) (Figure 12).

Table 15: Clinical and hematological characteristics of CML patients

N (%)
Groups
CML Patients UT (%) TNR (%) TR (%)

Total 61(100) 9(14.8) 12(19.7) 40(65.5)
Gender
Male 40(65.5) 7(17.5) 5(12.5) 28(70)
Female 21(34.5) 2(9.5) 7(33.4) 12(57.1)
Age
≤46 30(49.2) 5(16.7) 6(20) 19(63.3)
>46 31(50.8) 4(13) 6(19.3) 21(67.7)
RBC

Within Normal RBC count 6(9.8) 0(0) [0] 2(33.4) [17%] 4(66.6)
[10%]
Abnormal RBC count 55(90.2) 9(16.4)
[100%] 10(18.2) [83%] 36(65.4)
[90%]
WBC
Within Normal WBC count 47(77) 1(2.1) [11%] 11(23.4)
[92%] 35(74.5)
[88%]
Abnormal WBC count 14(23) 8(57.1)
[89%] 1(7.2)[8%] 5(35.7)
[12%]
Platelet
Normal platelet count 44(72.13) 4(9) [44%] 7(15.9)
[58%] 33(54)
[83%]
Abnormal platelet count 20(32.78) 5(25)[56%] 5(25) [42%] 10(50) [17%]
Hemoglobin (Hb)
Within Normal Hb level 10(16.2) 1(10)[11%] 1(10) [8%] 8(80) [20%]
Abnormal Hb level 53(86.8)) 8(15)[89%] 11(20.7) [92%] 32(60.3)
[80%]
Hematocrit (HCT)
Within Normal HCT level 14(23) 4(28.6) [44%] 2(14.3)
[17%] 8(57.1)
[20%]
Abnormal HCT level 47(77) 5(10.7) [56%] 10(21.3)
[83%] 32(68)
[80%]

UT- Untreated, TNR- Treatment Non Responders (MR 0, 1, 2), TR- Treatment Responders (MR 3, 4.5, 5)

All the CML patients in our study were positive for Philadelphia chromosome [t(9;22)] and the resulting BCR::ABL1 fusion protein at diagnosis. Upon analyzing the hematological findings within our patients, we found that majority of the patients who didn't receive any prior therapy (UT), had abnormal hemoglobin levels (89%), RBC counts (100%) and leukocytosis (89%). We analyzed the patients who received TKI therapy and found that majority of these patients had anemia (mean Hb) and abnormal hematocrit levels (mean HCT). When we compared the hematological profiles of TR and TNR group we observed that treatment non responders presented more often with thrombocytopenia as compared to TR group. We also observed that total leukocyte count, total RBC count, Hb levels and hematocrit were not very useful in differentiating TR from TNR group. (Table 15).

All 9 UT patients underwent qualitative BCR::ABL1 reverse transcriptase PCR analysis to determine the BCR::ABL1 transcript type. All the patients (61) were determined to harbor BCR::ABL1 Major (p210) transcript at diagnosis. None of our patients had minor or micro BCR::ABL1 transcripts. Following this we performed quantitative real time PCR (qPCR) based analysis for BCR::ABL1 transcript to reveal the measurable residual disease status of our study group patients. We calculated the NCN % and subsequently determined the NCN (IS)% utilizing the kit provided conversion factor (CF= 0.733). We detected a significantly higher number of BCR::ABL1 major fusion copies among the UT group. We also observed higher BCR::ABL1 major fusion copies in the TNR group while minimal copy numbers were detected in the TR groups. Thus, we observed a decreasing trend in average fusion copies, with the lowest average observed in the TR group (Figure 13).

Similarly, when comparing NCN% and IS values between different groups, we found UT group having the highest NCN and IS%, followed by TNR group. Minimum or undetected NCN and IS % was reported in the TR group. Accordingly, the average %NCN and % IS-NCN levels displayed a progressive decrease, indicating a move towards major molecular response, in the TR group when compared to the other two groups (UT and TNR groups) (Figure 14).

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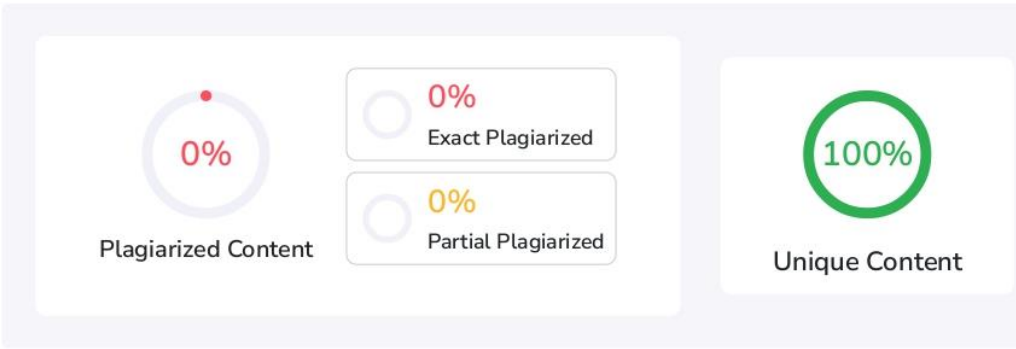

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Correlation of MBCR-ABL1 with various hematological parameters among UT, TNR and TR groups of CML patients

UT patients with higher expression of BCR::ABL1 copies and higher NCN and NCN IS % consistently presented with abnormal WBC counts as compared to patients who received treatment with TKI. We also observed that TR patients consistently presented with lower NCN and IS% and also had lower incidence of abnormal leukocyte counts. On the other hand, CML patients with abnormal platelet counts had higher levels of % IS-NCN of MBCR-ABL1 in UT and TNRs (Figure 12).

Utility of qPCR for determining therapeutic response among patients receiving TKI

Quantitative PCR (qPCR) is an efficient and reproducible technique for evaluating patient response in those given Tyrosine Kinase Inhibitors (TKIs) for disorders such as Chronic Myeloid Leukemia (CML). Using qPCR to evaluate MRD is a means of detecting low numbers of leukemic cells, through quantification of BCR-ABL1 fusion transcripts. This is especially useful as it can detect minimal residual disease in amounts that other methods would fail to identify. Tracking BCR-ABL1 over time by qPCR is an objective modality for assessing reduction in BCR-ABL1 transcripts that directly correlates to TKI therapy effectiveness.

qPCR is also used to detect early relapse. The ability of qPCR to detect rising levels of BCR-ABL1 prior to any clinical symptoms of relapse can offer clinicians an early opportunity to intervene and alter clinical management - thus potentially affecting the patient outcome. Therefore, qPCR has great utility not only in response assessment, but also in early prediction of relapse. qPCR also allows for comparison between different TKIs and thus speeds the avenue to identifying the optimal drug for the individual patient, especially in the case of less-than-optimal initial TKI response. In all, qPCR is indispensable in guiding treatment decisions and improving long-term prognosis by providing precise, real-time data on disease response and relapse risk.

Type of TKI received-

• Table 17: BCR-ABL: ABL Levels by RQ-PCR on Imatinib therapy.

NCN –IS (%) Number of patients at 6 months Number of patients at 12 months Number of patients > 12 months

>10% 07 02 04
<10-1% 02 0 01
<1-0.1% 0 0 11
<0.1-0.01% 0 0 09
<0.01-0.001% 0 0 08
<0.001% 0 0 17

• Table 18: Analysis of MMR status of Study group
Time point Optimal Warning Failure
Baseline 0 01 03
3 months 0 01 03
6 months 0 0 03
12 months 0 0 02
>12 months 40 0 07
• Table 19: History of Drug escalation/ Drug change
TNR TR
Drug escalation 12 37
Drug change 0 03

• Table 20: History of TKD mutation analysis
Number of patients
TKD mutation present 01
TKD mutation absent 02
Total number of patients tested 03
• Table 21: MRD trend in the study group among patients receiving TKI.
Therapy dynamics TR TNR
Ascending trend 01 03
Decreasing trend 22 05
Plateau 10 03
Irregular 07 01

• Table 22: Analysis of treatment dynamics of TNR group:
Never achieved MMR 9%
Loss of MMR 12%
Loss of CHR 79%

• Table 23: Analysis of treatment dynamics of TR group
Achieved MMR on 12 months 0%
Achieved MMR after 12 months 25%
Lost and again achieved MMR 0%
Achieved DMR 75%

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Total Words: 1000 **Total Characters:** 6970 **Plagiarized Sentences:** 0 **Unique Sentences:** 42 (100%)

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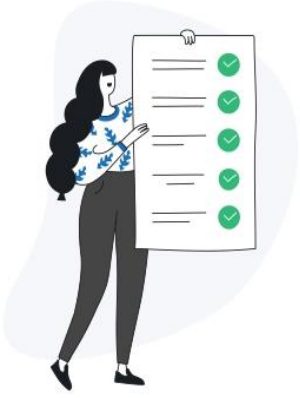
Discussion Chronic Myeloid Leukemia (CML) often manifests with vague, systemic complaints such as persistent fatigue, general malaise, and unintended weight loss. On examination, splenomegaly is a common finding, and as the disease progresses, symptoms related to leukostasis—such as headaches, visual impairment, or priapism—may emerge. Given the overlap in clinical features, particularly leukocytosis and splenic enlargement, differential diagnosis should include a range of myeloproliferative and infectious conditions, warranting a detailed diagnostic workup. Classification of CML relies on identifying its progression through three phases—chronic, accelerated, and blast—based on hematologic parameters, bone marrow analysis, and clinical presentation. Laboratory evaluation of CML typically reveals marked leukocytosis with a left shift in the myeloid lineage, accompanied by thrombocytosis and varying degrees of anemia. Bone marrow aspiration often demonstrates pronounced hypercellularity with an elevated granulocyte-to-erythroid (G:E) ratio and an expansion of myeloid precursors. A definitive diagnosis is established by identifying the Philadelphia chromosome (Ph), resulting from the t(9;22) (q34; q11) translocation, or its molecular counterpart, the BCR::ABL1 fusion gene. Cytogenetic techniques such as conventional karyotyping and FISH are essential for detecting this chromosomal abnormality. In addition, molecular assays like quantitative PCR (qPCR) and reverse transcription PCR (RT-PCR) enable sensitive quantification of BCR::ABL1 transcript levels, facilitating both disease monitoring and assessment of therapeutic response. qPCR-based MRD is considered a cornerstone of modern CML management which is essential for tailoring therapy, assessing prognosis, and enabling potential treatment-free approaches. In the present study, the median age of the study group was found to be 46 years. Similar studies have reported that the median age at diagnosis of CML in Asia and Africa is below 50 years, indicating a relatively lower age at onset in these regions[50]. Moreover, in current study, among 61 patients, 40 (65.5%) were males and 21 (34.5%) were females, indicating a male predominance in CML incidence. This observation aligns with previous findings by Amin et al (2021), Radhi et al (2023) and Kumar et al (2019)[26][5][51]. The male predominance may be attributed to the higher prevalence of haematological neoplasms in males, potentially influenced by genetic and hormonal disparities. In addition, a gender disparity might exist in our society due to which males regularly can afford diagnostic investigations as compared to females. The comparison across untreated, treatment non-responders, and treatment responder groups reveals distinct averages for various haematological parameters. Specifically, the untreated group exhibits an average RBC count of 3.35 x 103 cells/µl, while the treatment non-responders and responder groups show averages of 3.65 x 103 cells/µl and 3.75 x 103 cells/µl, respectively. WBC counts are measured at 97.74 x 103 cells/µl, 6.14 x 103 cells/µl, and 6.55 x 103 cells/µl for UT, TNR and TR groups, respectively. Platelet counts follow a similar trend with values of 243.11 x 103 cells/µl, 195.6 x 103 cells/µl, and 161.68 x 103 cells/µl in UT, TNR and TR groups, respectively. Haemoglobin levels are recorded at 10 g/dl, 11.3 g/dl, and 11.3 g/dl,

while HCT levels are measured at 32.6%, 32.8%, and 35.6% in UT, TNR and TR groups, respectively. Statistical analysis indicates significant differences in WBC count (p=0.0001) between treatment TNRs and TRs, while platelet count (p=1) RBC count (p=0.17), Hb level (p=0.16), and HCT level (p=0.15) do not exhibit significant variations among the groups. Analysing the haematological parameters like RBCs, WBCs, platelets, haemoglobin count, and haematocrit count, this study aligns with previous research. Specifically, studies by Pinto et al (2021), Ting et al (2017), Karaosmanoglu et al (2023) and Kumar et al (2019)[52][46] [24][5]found no significant difference in median RBC levels between patient groups. Regarding WBC levels, present results correspond with those of Amin et al (2021), Arun et al (2017), Karaosmanoglu et al (2023), Kumar et al (2019) and Bhatti et al (2012)[26][53][24][5][54], which showed a high significant difference between patients. However, concerning haemoglobin level, current results contradict those of Kumar et al. (2019), Bhatti et al (2012) and Granatowicz et al (2015)[5][54][55] which demonstrated a significant difference between patient and control groups. Lastly, present study aligns with previous research by Pinto et al (2021), Ting et al (2017), Karaosmanoglu et al (2023) and Kumar et al (2019)[52][46][24][5] indicating no significant difference in haematocrit levels between patient groups. The UT group shows an average LDH level of 3139.4 U/L, while LDH data for the TNR and TR groups was unavailable. This also points to the fact that haematological and biochemical assays (e.g. LDH) are not sufficient to reflect the MRD status in haematological neoplasms, and thus requires cytogenetic and molecular MRD assessments to manage these patients better. On assessing %NCN and %IS-NCN counts among three patient groups (UT, TR and TNR), untreated patients (UT) exhibit the highest levels of both measures, with averages of 57.65 and 36.35, respectively. This is followed by treatment non-responders and treatment responders, with significantly lower averages of 9.7/7.1 and 0.0038/0.0032, respectively. These findings strongly indicate the impact of treatment response on %NCN and %IS-NCN levels, showcasing an inverse correlation; as treatment response decreases, the levels of %NCN and %IS-NCN increase. Hence, a lower treatment response corresponds to higher %NCN and %IS-NCN levels, underscoring the importance of treatment efficacy in patient management. Similar findings have been reported by Jethva et al (2022) and Son NT et al (2021) [56][57] who analysed 50 Indian and 172 Vietnamese CML patients respectively. This further establishes the importance of quantitative PCR tests in segregating CML patients according to their therapeutic response. As noted above, this study also demonstrated that Indian generic imatinib mesylate (IM) is a viable, cost-effective, clinically advantageous option for CML-CP with treatment outcomes comparable to the original branded molecule, Gleevec. This small cohort of study participants also demonstrates how central molecular monitoring with real-time quantitative PCR (RQ-PCR) can be influential in determining the presence of minimal residual disease, and early log reductions in BCR-ABL transcript levels, especially a 1-log reduction at three months, predicted MMR (major molecular response) at the 12-month milestone. In the referenced study between the evaluable patients at the 12 to 24 months milestone, of which % achieved MMR, where 24.5% achieved



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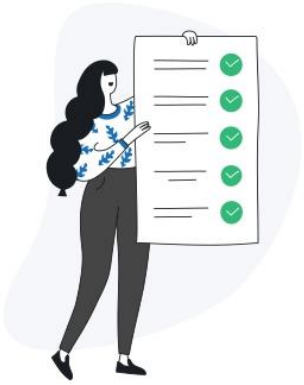
Conclusion

This study offers a thorough look at the role of qPCR and other commonly available laboratory techniques which find application in prognostication and therapeutic monitoring among CML patients. Our results confirm that CML mainly affects men and tends to affect patients at a younger average age in developing countries, which aligns with wider demographic patterns. We analysed the current international diagnostic and therapeutic guidelines in CML and integrated them in our study process. There's a notable inverse relationship between how well patients respond to treatment and certain molecular markers (%NCN and %IS-NCN), highlighting the need for effective therapies and careful molecular monitoring. Haematological factors, especially the white blood cell (WBC) count, proved to be solid indicators of how the disease progresses and how patients respond to treatment. Importantly, this study points out the effectiveness and cost-effectiveness of Indian generic imatinib mesylate (IM), making it a strong option for first-line treatment in settings with limited resources. The use of RQ-PCR for early detection of molecular response and tracking minimal residual disease further enhances its role in tailored disease management. However, the occurrence of molecular relapse in patients who initially achieved a complete molecular response highlights the importance of ongoing monitoring after treatment. This study also emphasises the need for reproducible and standardised MRD protocols to better manage these patients. Overall, this study provides valuable insights into the role of molecular diagnostic in managing CML and emphasizes the need for continuous assessment of both clinical and molecular factors to improve patient outcome.



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